Influence of the lab adopted natural diet, environmental, and parental microbiota on life history and metabolic phenotype of *Drosophila melanogaster* larvae.

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Abstract:

Obesity is an increasing worldwide epidemic and contributes to physical and mental health losses. The development of obesity is caused by multiple factors including genotype, hormonal misregulation, psychological stress, and gut microbiota. Our project investigated the effects produced by microbiota community, acquired from the environment and horizontal transfer, on traits related to obesity. The study applied a novel approach of raising *Drosophila melanogaster* from ten, wild-derived genetic lines (DGRP) on naturally fermented peaches, thereby preserving genuine microbial conditions. Our results indicated that larvae raised on the natural and standard lab diets were significantly different from each other in every tested phenotype. In addition, sterilized larvae raised on the autoclaved peach diet, therefore exposed to natural nutritional stress but lacking natural microbiota community, were associated with adverse phenotypes such as low survival rate, longer developmental time, smaller weight, and elevated triglyceride and glucose levels. Our findings suggested that frozen peach food provided nutritional conditions similar to the natural ones and preserved key microbial taxa necessary for survival and development of *Drosophila* larvae. The presence of parental microbiota did not produce a significant effect on any of the tested phenotypes when larvae were raised on the lab diet. Contrarily, on the peach diet, the presence of parental microbiota increased the weight and development rate, even if the original peach microbiota were still present. In addition, we found that larvae raised on the peach diet formed a microbial community distinctive from larvae raised on the lab or peach autoclaved diets. The effect that individual microbial taxa produced on the host varied significantly with changing environmental and genetic conditions, occasionally to the degree of opposite correlations.

40 **Introduction**

41 The holobiont theory states that a host and its commensal microbiota possess a metagenome that
42 expresses a synergistic phenotype, which is subjected to evolutionary forces as one complex
43 organism (Rosenberg et al., 2010). A phenotype of this unit could be varied by genome
44 modifications of the host, as well as its commensal bacteria. Metagenomic changes induced by
45 bacteria have more potential for genetic variability and could arise by altering dominant species
46 of bacteria, as well as acquisition of new strains of microorganisms from an environment
47 (Bordenstein and Theis, 2015).

48 Obesity is a worldwide epidemic that continues to grow and contributes to the development of
49 various diseases, including but not limited to: type two diabetes mellitus, stroke, asthma,
50 arthritis, coronary heart disease, arterial hypertension, all components of metabolic syndrome
51 (Seganfredo et al., 2017, Wahba and Mak, 2007). Obesity does not only induce health risks but
52 also is an important social factor. Stereotypically, the major cause of obesity is considered to be
53 overeating and lack of exercise. Obese individuals are often stigmatized as lazy and
54 unsuccessful, making them vulnerable to discrimination (Puhl and Heuer, 2010). Contrary to the
55 stereotypic view, the development of obesity and metabolic syndrome is caused not only by
56 excessive calorie intake and lack of physical activity but also by genotype, epigenetic factors,
57 sleep deprivation, malfunction of endocrine system, psychological stress, and gut microbiota
58 (Seganfredo et al., 2017, Han and Lean, 2016).

59 Gut microbiota is one of the most important factors shaping metabolic phenotype and, as the
60 consequence is a key element in the development of metabolic and autoimmune diseases, cancer,
61 and asthma (Read and Holmes, 2017, Leitão-Gonçalves et al., 2017). Alterations in gut

62 microbiota biodiversity and community structure are correlated with the development of the
63 obese phenotype (Turnbaugh et al., 2006, Flint et al., 2017). Transfer of the microbiota from an
64 obese to a lean, axenic (microbiota free) individual significantly increases weight gain and
65 adiposity, compared to axenic mice colonized with microbiota community from a lean organism
66 (Ridaura et al., 2013, Tilg and Moschen, 2016, Turnbaugh et al., 2006). These results suggest
67 that obesity can be transferred from one individual to another; therefore, exhibiting some
68 characteristics of an infectious disease (Tilg and Moschen, 2016).

69 During colonization of a fruit, *Drosophila* inoculates the substrate with their microbiota (Morais
70 et al., 1995). In addition, when females deposit embryos on a substrate, parental microbiota
71 resides on the chorion of the egg (Ryu et al., 2008). The initial microbiota is gained by the first
72 instar larvae through consumption of the chorion (Ryu et al., 2008). Later, during feeding, larvae
73 acquire additional microbiota from the environment, representatives of which remain in the
74 larval and pupal intestine until eclosion of the adult fly (Ridley et al., 2012). Wong et. al (2015)
75 showed that parental microbiota transferred with the chorion of the egg could modify the
76 microbial community composition in a food substrate and in the offspring. In addition, the
77 transfer of axenic *Drosophila* on food substrate would change the food microbial community to
78 resemble the symbiotic microbiota composition that would develop in the host (Wong et al.,
79 2015).

80 Symbiotic microbiota play an important role in *D. melanogaster* development and metabolic
81 phenotype. Axenic flies have a longer development time, lower weight, protein, and glycogen
82 content but higher free glucose and triglyceride levels (Newell and Douglas, 2014b, Dobson et
83 al., 2015, Huang and Douglas, 2015). There are similar negative phenotypic effects on axenic *D.*
84 *melanogaster* and on those being raised on harmful diets such as high sugar and high fat (Newell

85 and Douglas, 2014a, Huang and Douglas, 2015, Reed et al., 2014, Birse et al., 2010, Dew-Budd
86 et al., 2016). Although axenic *Drosophila* consume less food, their energy storage indices
87 (triglyceride, glucose, glycogen, and trehalose levels) stay significantly higher than that of
88 conventional flies (Wong et al., 2014). In addition, the presence of the commensal microbiota
89 allows *Drosophila* to maximize their lifespan and reproductive output (Leitão-Gonçalves et al.,
90 2017). Dobson et. al (2015) showed that the abundance of specific bacterial taxa is associated
91 with a change in metabolic phenotype: *Acetobacter*, *Gluconobacter* and *Komagataibacter* are
92 negatively correlated with a fly's energy storage index. *Lactobacillus* is positively associated
93 with triglyceride concentration and *Achromobacter* and *Xanthomonadaceae* have a positive
94 correlation with glycogen levels (Chaston et al., 2014, Chaston et al., 2016). In addition,
95 *Drosophila* microbiota varies across genetic backgrounds which makes it possible to establish
96 associations between a host's genes and microbiota dependent metabolic responses as well as
97 particular symbiotic species (Chaston et al., 2016, Dobson et al., 2015, Early et al., 2017).

98 Symbiotic microbiota allow *Drosophila* to overcome the nutritional limitation of their diet. Shin
99 et. al (2011) showed that axenic larvae raised on a casamino acid diet experienced a 90% body
100 size reduction and were not able to survive to form pupae. However, the presence of only one
101 bacterial species, *Acetobacter pomorum,* could restore survival and the normal rate of larval
102 development via induction of the hosts' insulin-like growth factor signaling (Shin et al., 2011).
103 Leitão-Gonçalves et. al (2017) demonstrated that axenic flies express a strong preference for
104 yeast-rich food due to their demand for essential amino acids. *A. promorum* and several
105 *Lactobacilli* species are able to suppress the yeast appetite in *Drosophila* and shift the flies'
106 nutritional preference toward high-sugar concentration diets (Leitão-Gonçalves et al., 2017).
107 Their change in nutritional preference may be explained by competition between the host and its

108 symbiotic microbiota for available sugars and through production of essential amino acids by the
109 microbial community (Leitão-Gonçalves et al., 2017).

110 Microbiota composition of lab dwelling *Drosophila* primarily consists of Acetobacteraceae,
111 Enterococcaceae, and Lactobacillaceae families (Chandler et al., 2011, Early et al., 2017).
112 Within these families, the influence of *Acetobacter tropicalis*, *Enterococcus faecalis*,
113 *Lactobacillus brevis*, and *Lactobacisul plantarum* on a host's metabolic phenotype have been
114 studied more than others (Early et al., 2017, Shin et al., 2011, Huang and Douglas, 2015).
115 However, the microbiota of wild fly populations may differ in the diversity and the abundance of
116 dominant species (Chandler et al., 2011). In the lab*, Drosophila* raised on fruits still exhibit a
117 more complex and diverse community of symbiotic microbes compared to conventionally raised
118 flies (Vacchini et al., 2017). Lab food preservatives, especially methylparaben sodium salt
119 (moldex), largely contribute to the difference between natural and laboratory associated
120 microbiota communities (Tefit et al., 2017). Therefore, studying the evolutionary relationship of
121 *Drosophila* and its microbiota, as well as the symbiont's influence on fly's metabolic phenotype
122 only on standard lab microbiota, may be insufficient to understand the natural relationship and
123 co-evolution of the fly and its microbiota.

124 With this work we wanted to address a series of specific questions and hypotheses:

125 **1)** How does a natural diet with a naturally occurring and/or maternally inherited community of
126 microbes influence the life history and metabolic phenotypes of flies relative to a standard lab
127 diet? Is there genetic variation in the phenotypic response to nutritional change and dietary and
128 parental microbiota availability?

129 **1.1** We hypothesized that larvae raised on a natural diet will exhibit different life history
130 traits and metabolic phenotypes, comparing with larvae raised on a standard lab diet.

131 **1.2** We hypothesized that the presence of a maternally transmitted microbiota will
132 significantly impact larvae phenotypes, and that this impact may vary across dietary
133 treatments.

134 **1.3** Given prior findings on the roles of genetic variation on metabolic phenotypes, we
135 hypothesized that there is genetic variation in phenotype that interacts with the dietary
136 conditions and the availability of maternally transmitted microbiota.

137 **2)** Will the symbiotic microbiota community of the larvae raised on the natural diet be different
138 from the lab food raised larvae? Will the presence of maternally inherited microbiota influence
139 the formation of microbial communities? Is the microbiota community variable across host
140 genotypes?

141 **2.1** We hypothesized that the gut microbial community composition and diversity will
142 vary substantially across both dietary and parental microbiota conditions.

143 **2.2** We hypothesized that the maternally transmitted microbiota will have "founder
144 effects" in the formation of the larval gut microbiome.

145 **2.3** We hypothesized that the composition of the microbial community will exhibit
146 variation with host genotype.

147 **3)** Will specific microbial taxa and/or microbiota communities as a whole influence the larvae
148 phenotypes differently across diets and genotypes?

149 **3.1** We hypothesized that some microbial taxa will have consistent correlations with host
150 phenotype across diets and treatments while others will have a diet or treatment specific
151 relationship.

152 ## Materials and Methods

153 **Diets preparation**

154 On August 28th, 2017, we put approximately 200 peaches outdoors and allowed them to decay
155 for six days. On September 3rd 2017, the fruits were collected, manually ground, and stored in
156 freezers at -20 °C. Peach food (PR) preparation protocol was the following: we allowed
157 approximately one liter of the peach food to thaw, homogenized it with an immersion blender,
158 and distributed it into vials, with approximately 10 ml of food per vial. In order to prepare
159 autoclaved peach food (PA), vials containing peach food were autoclaved for 25 min at 121 °C.
160 Regular *Drosophila* lab food (R) was cooked according to the protocol described in previous
161 works (Dew-Budd et al., 2016, Mendez et al., 2016).

162 To ensure that the autoclaved peach food did not contain any live microorganisms, we used 1g of
163 autoclaved and non-autoclaved peach materials and diluted them in 9 ml of sterile Phosphate-
164 buffered saline solution (PBS) (Leitão-Gonçalves et al., 2017, Carvalho et al., 2005). Then, we
165 performed a serial dilution in PBS to get food dilutions (Leboffe and Pierce, 2012). We mixed 1
166 ml of each dilution with standard methods agar (Criterion) via the pour plate method (Leboffe
167 and Pierce, 2012). The agar was prepared according to the manufacturer's directions. Samples
168 were incubated at 35 °C for 48 hours (Maturin et al., 2001). The independent variable for the diet
169 component will be referred to as D.

170 **Drosophila stocks and husbandry**

171 We used 10 naturally derived genetic lines created by the DGRP2 project: 142, 153, 440, 748,
172 787, 801, 802, 805, 861, and 882 (Mackay et al., 2012, Huang et al., 2014). Stocks were
173 maintained at constant temperature, humidity and light/dark cycle on a molasses-based lab diet
174 as described in previous works (Reed et al., 2014, Dew-Budd et al., 2016, Mendez et al., 2016).
175 The independent variable for the genetic component will be referred to as G.

176 **Drosophila embryos sterilization**

177 In order to remove parental microbiota, we sterilized ~12-hour old embryos with subsequent
178 two-minute washes in 2.5% active hypochlorite solution, 70% ethanol solution, and sterilized
179 distilled water (Leitão-Gonçalves et al., 2017, Carvalho et al., 2005). After sterilization, embryos
180 were placed on the apple agar plates, and incubated until the first instar stage under fly-rearing
181 conditions described above (Ashburner, 1989). The non-sterilized control embryos (NS) were
182 allowed to develop for ~24 hours (until the 1st instar larvae stage) on the apple agar plates, on
183 which they had been deposited. In order to demonstrate that sterilized embryos did not possess
184 parental microbiota, 20 sterilized 1st instar larvae were collected and grinded in 200 ul of the
185 sterile PBS using a mechanical homogenizer. The resulting mixtures were plated on nutrient and
186 standard method agars (Criterion). The agars were prepared according to the manufacturer's
187 directions. Plates were incubated for 48 hours at 35 °C (Maturin et al., 2001). NS larvae were
188 used as the positive control according to the same procedure. The independent variable for the
189 sterilization treatment component will be referred to as T.

190 **Larvae rearing and collection**

191 In three separate time periods (~ 30 days apart), we put 50 sterilized and non-sterilized larvae of
192 each genetic line in at least three vials of PA, PR and R food, each. The independent variable for
193 the time period (round) component will be referred to as R. Larvae were allowed to develop until
194 the late third instar wandering stage (when they stopped moving but before the pupation started)
195 and then collected in micro centrifuge tubes with sterile Ringer's solution (Ashburner, 1989).
196 Each vial was checked for the presence of larvae, at the right developmental stage, four times per
197 day at 9 am, 11 am, 2:30pm, and 5 pm for 18 days after larvae colonization. Larvae were
198 inspected for the presence of any damage (damaged ones were sorted out), cleaned with at least
199 two washes in a sterile Ringer's solution, and stored in the Ringer's solution at -20 °C in
200 Eppendorf tubes with 10 larvae per tube.

201 **Measuring Experimental Phenotypes**

202 **Survival.** The number of larvae collected per vial was summed and used to evaluate the number
203 of larvae that survived till the late 3rd instar stage. **Developmental rate**: The developmental rate,
204 in days, was calculated for each larva individually, from the day it was put in the food vial to the
205 collection date. We then calculated median developmental time per vial and used it in our
206 statistical analysis (Ridley et al., 2012). **Weight**: In order to measure the dry weight, larvae were
207 taken from the -20 °C freezer, allowed to reach room temperature and placed in a VWR standard
208 oven at 37 °C overnight. After drying, each larva was weighed individually using Mettler Toledo
209 XS 105 microbalance. Weights were recorded with LabX direct software v. 2.2. **Triglyceride**:
210 With the exception of four samples (due to low survival of larvae of certain D/G/T/R
211 combinations), we homogenized 10 larvae per sample to determine total triglyceride
212 concentration using the Sigma Triglyceride Determination Kit (Clark and Keith, 1988, De Luca
213 et al., 2005, Reed et al., 2010, Dew-Budd et al., 2016). Results were adjusted to represent the

214 average triglyceride level per mg of dry larval weight. **Protein**: Protein levels were quantified
215 using the Bradford's method with 10 homogenized larvae per sample (with the exception of 3%
216 of the samples in which we used one to nine larvae, due to especially low survival rates of the
217 specific groups) (Bradford, 1976, Dew-Budd et al., 2016). Protein levels were averaged to
218 represent the protein concentration per mg of dry larvae weight. **Glucose**: For most of the
219 samples, combined trehalose and glucose concentrations were quantified via homogenization of
220 10 larvae (with the exception of 5% of the samples in which we used four to nine larvae) with
221 subsequent overnight incubation in 1 μg/mL trehalase solution and further application of the
222 Sigma Glucose Determination Kit (Rulifson et al., 2002, Reed et al., 2014, Dew-Budd et al.,
223 2016). Glucose levels were averaged and adjusted to represent the amount of glucose per mg of
224 dry weight.

225 To assess the triglyceride, protein and glucose levels in the diets, we used freshly unfrozen food
226 and unfrozen food that was incubated in the food vials for seven days at 25 °C, in the same
227 incubator as the experimental fly stocks, where larvae development was taking place. For the
228 food assays, we used the same procedure as for the larvae but with ~12.35 mg of the food
229 sample. For the glucose assay, instead of incubating samples in trehalase, we incubated them in
230 100μl of 1 mg/μl solution of invertase (to convert sucrose into glucose) (Ward's Natural Science)
231 overnight at 37 °C. For analysis, the results were adjusted to represent the amount of the
232 measured compound per mg of the sample.

233 **DNA extraction and sequencing**

234 DNA was extracted from 10 larvae with the Qiagen blood and tissue DNA extraction kit
235 according to the standard protocol, with overnight incubation of the samples in proteinase K at

236 56 °C. DNA extractions were used for sequencing the V4 region of the 16S ribosomal RNA
237 subunit (16S rRNA), which was performed in the Microbiome Core Facility of The University of
238 Alabama in Birmingham, AL according to the previously published method on the Illumina
239 MiSeq platform (Kumar et al., 2014).

240 Trimmomatic-0.36 (Bolger et al., 2014) was used to process demultiplexed DNA sequences.
241 Standard Illumina-specific barcode sequences, all sequences with less than 36 bases, and leading
242 and trailing low-quality bases were all removed using the default settings of the Trimmomatic-
243 0.36 program. The USEARCH-fastq_mergepairs tool was used to combine forward and reverse
244 readings. All reads with an expected error greater than 1 were removed, as well as chimeric reads
245 and singletons. Combined readings without a merging pair were filtered using fastq_filter
246 command. The -cluster_otus tool was used to cluster readings into operational taxonomic units
247 (OTUs) with 97% identity. The -unoise3 tool was used to cluster readings into zero-radius OTU
248 (ZOTU) 100% identity. OTUs were then designated with the lowest taxonomic rank using the
249 UCULT algorithm implemented in QUIME 1.9.1 (Caporaso et al., 2010b, Edgar, 2010) along
250 with SILVA reference database version 132 (Quast et al., 2012). Using SILVA v. 132 database,
251 PyNAST (Caporaso et al., 2010a) with default options was used for sequence alignment. The
252 phylogenetic trees of ZOTUs and OTUs were assembled using the default options of QUIIME
253 1.9.1 with the FastTree program (Price et al., 2009). Alpha and beta diversities were rarefied
254 with QUIIME 1.91 -single_rarefaction.py using the --subsample_multinomial option in order to
255 subsample the replacements. Rarefaction for all samples was performed to the depth of 4,500
256 readings. This was the lowest possible number of readings between samples.

257 **Statistical Analysis**

258 **Data transformation.** Normality tests, data transformations and statistical models were done
259 with JMP Pro 14.0. Phenotype measurements were tested for normality with the Shapiro-Wilk
260 test and an outlier box plot. Only larvae survival (**ST. 1**) showed normal distribution. Therefore,
261 all other phenotypic measurements data were transformed. We performed a cube root
262 transformation on the data for development rate and glucose by weight, a square root
263 transformation on data for weight and protein by weight, and a log transformation on data for
264 triglyceride by weight levels. The microbial abundance was log(x+1) transformed for all
265 parametric analyses.

266 **Microbial Diversity**. Alpha and beta diversities were computed in QIIME v. 1.9.1. To estimate
267 alpha diversity, we used Shannon, Simpson, and PD Whole Tree metrices. As all of the alpha
268 diversity indices were not normally distributed, we performed a pairwise comparison of them
269 with a Wilcoxon rank-sum test in R v. 3.5.1 with "matrixTests" package v. 0.17 (Bruno et al.,
270 2019). Beta diversity was estimated with Bray-Curtis and Weighted Unifrac distances. The
271 similarity between each sample's beta diversity distance was evaluated via hierarchical
272 clustering, applying a ward method for distance calculation, and visualized with a constellation
273 plot in JMP v. 14.0.

274 **Statistical modeling:** In order to evaluate the contribution of each variable and their interactive
275 effect on each phenotypic development, we used standard least squares model with model effects
276 to include Diet (D), Genotype (G), Sterilization Treatment (T) and their specific interactive
277 effect: D*G (diet by genotype), D*T (diet by treatment), G*T (genotype by treatment), D*G*T
278 (diet by genotype by treatment). In order to verify that the built model fits the data, a Lack of Fit
279 test was performed. If the time period of the experiment (R) and/or the variance between the
280 colorimetric assay runs (triglyceride, protein, and glucose) (P) produced a significant effect,

these variables were included in the model's effects, unless their addition caused the model to fail the lack of fit test. Thus, the models for larvae survival, development time, and weight were the following:

$$y_i = \beta_0 + \beta_1 D_{1i} + \beta_2 G_{2i} + \beta_3 T_{3i} + \beta_4 (D * G)_{4i} + \beta_5 (D * T)_{5i} + \beta_6 (G * T)_{6i} + \beta_7 (D * G * T)_{i7} + \varepsilon_i$$

For triglyceride by weight:

$$y_i = \beta_0 + \beta_1 D_{1i} + \beta_2 G_{2i} + \beta_3 T_{3i} + \beta_4 (D * G)_{4i} + \beta_5 (D * T)_{5i} + \beta_6 (G * T)_{6i} + \beta_7 (D * G * T)_{i7} + \beta_8 P_{8i} + \varepsilon_i$$

And for protein and glucose by weight:

$$y_i = \beta_0 + \beta_1 D_{1i} + \beta_2 G_{2i} + \beta_3 T_{3i} + \beta_4 (D * G)_{4i} + \beta_5 (D * T)_{5i} + \beta_6 (G * T)_{6i} + \beta_7 (D * G * T)_{i7} + \beta_8 P_{8i} + \beta_9 R_{9i} + \varepsilon_i$$

Where y_i is the response, β values are constants, and ε_i is a random error term.

All models for interactive effects of diet, genotype, and treatment were done with all 10 genetic lines, with the exception of glucose which was done without 861 due to the low survival rate of this genetic line.

In order to verify that the built model fits the data, a Lack of Fit test was performed. Only the models with non-significant lack of fit p-value were kept and used for an evaluation. To assess the pairwise difference between diets (R vs PR and PR vs PA) and treatments (NS vs S), we used the least square model with one main explanatory variable of interest (Diet or Treatment). We also included time period and assay plate variance as additional explanatory variables if they

301 produced a significant effect. We then used Post-Hoc pairwise comparisons to evaluate the
302 Student's t-test.

303 **Microbial abundance.** In order to evaluate if the diet and treatment could serve as categorical
304 predictors for classification of the larvae microbial samples, we performed discriminant analysis
305 at phylum, class, order, family, and genus taxonomic levels, as well as at the level of individual
306 ZOTUs. The results were visualized with a canonical plot in JMP v. 14.0 (JMP manual). For the
307 10 most abundant representatives of each taxonomic level, we applied the linear covariance
308 method for the discriminant analysis which allowed us to visualize the covariates in the form of
309 rays. Using this method allowed us to represent which of them drove the separation of the
310 clusters (JMP manual). When we ran the analysis with all identified taxa, we applied a wide
311 linear method for the discriminant analysis. To compare the abundance of microbial taxa
312 between the diets and treatments, we performed a Wilcoxon test as described above. The p
313 values were adjusted for the false discovery rate with Benjamini-Hochberg correction and added
314 to data tables as FDR p. The threshold for the value of FDR p that should be considered
315 significant could be subjective and vary from 0.25 to 0.05 among microbiology studies (Wu et
316 al., 2011, Bruce-Keller et al., 2015). To evaluate the interactive effect of the variables on the
317 abundance of each identified microbial taxa, we used the three-way interaction model.

318 $$y_i = \beta_0 + \beta_1 D_{1i} + \beta_2 G_{2i} + \beta_3 T_{3i} + \beta_4 (D * G)_{4i} + \beta_5 (D * T)_{5i} + \beta_6 (G * T)_{6i}$$
319 $$+ \beta_7 (D * G * T)_{i7} + \beta_8 R_{8i} + \varepsilon_i$$

320 In order to identify the correlations between phenotypes and microbial abundances, we found the
321 average phenotype for each combination of diet, genetic line, treatment, and round and aligned it
322 with the microbial sequences of the corresponding combination of independent variables.

323 Spearman's rank correlation between the microbial abundances and tested phenotypes was
324 calculated with Hmisc v. 4.3-0 in R v. 3.5.1, with the adjustment of p values for FDR p as
325 described above. We also tested the possible interactive effect of each identified microbial taxa
326 and one of the independent variables on the formation of the tested phenotypes according to the
327 formula:

328 $$y_i = \beta_0 + \beta_1 x_{1i} + \beta_2 x_{2i} + \beta_3 (x_{1i} * x_{2i})_{3i} + \beta_4 R_{4i} + \varepsilon_i$$

329 where, x_1 was the abundance of the microbial taxa, x_2 was one of the independent variables
330 (D, G, or T) and R was time component. Development, weight, triglyceride, protein, and
331 glucose were normalized with log, square root, log, log, and cube root, transformations
332 respectively.

333 Results

334 1) The influence of a natural diet with a naturally occurring and/or maternally inherited 335 community of microbes on the life history and metabolic phenotypes of flies relative to a 336 standard lab diet

337 **1.1** Larvae raised on a natural diet exhibited different life history traits and metabolic phenotypes
338 compared to larvae raised on a standard lab diet. **Survival.** The number of larvae that survived
339 on the lab diet was significantly higher than the larval survival on a natural peach diet regardless
340 of sterilization (NS: p= 0.001, S: p= 0.0079) (Fig. 1A, Table 1.1, Sup. Table 1.1). The
341 undisturbed microbial community of the peach diet produced a significant positive effect on
342 larvae survival, when compared with the autoclaved peach diet for both sterilized and non-
343 sterilized larvae (NS: p= 0.0001, S: p< 0.0001) (Fig. 1A, Table 1.1, Sup. Table 1.1).

344 **Development rate.** Within both controlled and sterilized treatments, larvae developed faster on
345 the regular lab diet compared to the natural diet (NS: $p= 0.0002$, S: $p< 0.0001$) (Fig. 1B, Table
346 1.1, Sup. Table 1.1) and faster on the original peach food compared to the autoclaved diet (NS:
347 $p< 0.0001$, S: $p< 0.0001$) (Fig. 1B, Table 1.1, Sup. Table 1.1). **Weight.** Larvae raised on the lab
348 food were significantly heavier than those that were raised on the natural diet (NS and S: $p<$
349 0.0001) (Fig. 1C, Table 1.1, Sup. Table 1.1). Among the peach diets, larvae consuming the
350 autoclaved diet were significantly lighter (NS and S: $p< 0.0001$) (Fig. 1C, Table 1.1, Sup. Table
351 1.1), suggesting that under natural nutritional conditions, microbiota in the food substrate
352 facilitate growth and weight gain of the larvae. **Triglyceride.** Although fresh and incubated R
353 food had higher triglyceride levels than the PR food (both $p< 0.0001$) (Sup. Table 1.2, 1.3),
354 larvae raised on the PR diet had significantly higher triglyceride concentrations by weight than
355 those that were raised on a lab diet, independent of sterilization treatment (NS and S: $p< 0.0001$)
356 (Fig. 1D, Table 1.1, Sup. Table 1.1). Incubated autoclaved peach food had significantly higher
357 triglyceride content ($p< 0.0001$) comparing with regular peach food (Sup. Table 1.3) and
358 produced larvae with higher triglyceride by weight levels (NS and S $p< 0.0001$) (Fig. 1D, Table
359 1.1, Sup. Table 1.1). **Protein.** Independent of the treatment, larvae raised on the regular food had
360 higher protein by weight levels compared to larvae raised on peach food (NS: $p< 0.0045$, S: $p<$
361 0.0001) (Fig. 1E, Table 1.1, Sup. Table 1.1). Larvae raised on the PA diet had significantly
362 higher protein by weight levels compared to PR raised larvae, but only in the absence of parental
363 microbiota (NS: $p= 0.099$, S: $p< 0.0001$) (Fig. 1E, Table 1, Sup. Table 1.1). Evaluating the
364 difference in fresh food protein content, we found significantly higher protein concentration in
365 the regular lab diet compared to the peach diet ($p< 0.0001$) (Sup. Table 1.2). Between fresh
366 peach diets there was no significant difference (Sup. Table 1.2). However, after incubation,

367 autoclaved peach food had significantly less protein than regular peach food ($p= 0.0389$) (Sup.
368 Table 1.3). **Glucose.** Larvae raised on a lab food diet had significantly higher glucose levels than
369 larvae raised on the peach food diet (NS: $p< 0.0001$, S: $p< 0.0001$) (Fig. 1F, Table 1, Sup. Table
370 1.1). Larvae that consumed PR food had lower glucose by weight levels compared with larvae
371 raised on the autoclaved version, which was consistent with our 1.1 hypothesis (NS: $p= 0.0089$,
372 S: $p< 0.0001$) (Fig. 1F, Table 1, Sup. Table 1.1). Interestingly, the fresh peach food had a
373 significantly higher glucose concentration than the lab food ($p< 0.0001$) (Sup. Table 1.2).
374 However, after incubating the peach food, the concentration of glucose was lower in PR than in
375 both R ($p<0.0001$) and PA ($p< 0.0001$) diets (Sup. Table 1.3), suggesting a strong impact from
376 the live microbial community.

377 **1.2** We observed that the presence of a maternally transmitted microbiota significantly impacted
378 larvae phenotypes, and that impact varied across dietary treatments. **Survival.** The parental
379 microbiota did not produce a significant effect on the larvae's survival on the lab diet or the non-
380 autoclaved peach diet (Fig. 1A, Table 1.2, Sup. Table 1.4), but its presence significantly
381 enhanced the overall survival of larvae on the autoclaved peach diet ($p< 0.0001$) (Fig. 1A, Table
382 1.2, Sup. Table 1.4). This suggested the existence of microbial taxa that were necessary for a
383 successful transition of the larvae through the instar stages under natural nutritional conditions,
384 and that these microbial species could be picked up from the food substrate if available and/or
385 inherited maternally. **Development rate.** Presence of parental microbiota on the peach diet
386 reduced the number of days necessary for larvae to reach the third instar stage ($p= 0.0002$) and
387 autoclaved peach diet ($p< 0.0001$) but not on a regular lab diet (Fig. 1B, Table 1.2, Sup. Table
388 1.4), suggesting that under natural nutritional conditions, maternal microbes might influence the
389 developmental rate independent of the microbiota acquired from the food substrate. **Weight.**

390 Maternally inherited microbiota produced a significant positive effect on larval weight on all of
391 the tested diets (R: $p= 0.005$, PR: $p= 0.0003$, PA: $p< 0.0001$) (Fig. 1C, Table 1.2, Sup. Table
392 1.4). This indicated the universality of their influence on larval growth across food substrates.
393 **Triglyceride.** Parental microbiota did not influence the triglyceride levels significantly on any
394 diet (Fig. 1D, Table 1.2, Sup. Table 1.4). **Protein.** Evaluating the role of parental microbiota, we
395 observed that sterilized larvae had higher protein by weight levels but only on the PA diet ($p=$
396 0.0014) (Fig. 1E, Table 1.2, Sup. Table 1.4). This suggested that the core microbiota involved in
397 a natural metabolic phenotype formation might be inherited or acquired from the environment.
398 **Glucose.** The parental microbiota reduced the glucose by weight levels only on PA food ($p=$
399 0.001) (Fig. 1F, Table 1.2, Sup. Table 1.4), indicating that both parental and environmental
400 microbial taxa might be sufficient to reduce glucose levels in larvae.

401 **1.3** Evaluating the contribution of tested independent variables on larvae phenotypes, we
402 observed a genetic variation in most of the tested life history traits and phenotypes that interacted
403 with the dietary conditions and the availability of maternally transmitted microbiota. **Survival.**
404 All of the independent variables included in the model produced a significant effect on the
405 larvae's ability to survive until the late third instar stage (for diet, genetic line, treatment, diet by
406 genotype, and the interactive effect of diet by genetic line $p< 0.0001$, genetic line by treatment
407 $p= 0.0374$, and the interactive effect of diet, genetic line and treatment $p< 0.0009$). Out of the
408 tested variables, diet was the strongest predictor of survival (variance explained (VE) 28.4%),
409 followed by the interactive effect of the diet by treatment (VE= 8.02%) and genetic line (VE=
410 5.13%) (Table 1.3, Sup. Table 1.5). **Development.** The development rate of the larvae was
411 significantly influenced by diet ($p< 0.0001$), genotype ($p< 0.0001$), and treatment ($p< 0.0001$)
412 (Table 1.3). Among the specific interaction of these variables, only D*T ($p< 0.0001$) and G*T

413 (p= 0.0374) produced a significant effect on development (Table 1). Diet was the key factor that
414 influenced the time necessary for the larvae to reach the late third instar stage and explained
415 almost half of all variance (VE= 47.5%) followed by the genetic line (VE= 4.98 %, Table 1). The
416 combination of the rest of the variables was responsible only for 8.42 % of variation in
417 developmental time (Table 1.3, Sup. Table 1.5).

418 **Weight.** All of the independent variables, with the exception of D*T, produced a significant
419 effect on dry larval weight (p< 0.0001) with diet being the best predictor (VE= 31.4%), followed
420 by genotype (VE= 7.44%), and D*G interaction (VE= 2.88%) (Table 1.3, Sup. Table 1.5).
421 **Triglyceride.** Once again, diet explained the largest portion of variance (VE= 41.4%) across all
422 independent variables. The interactive effect of D*G was a better predictor of triglyceride levels
423 than the genotype (VE= 5.03% and 2.36%, respectively) (Table 1.3, Sup. Table 1.5). **Protein.** In
424 contrast with other measured phenotypes, the variance explained by the model was
425 predominantly evenly distributed across the independent variables, with genotype having the
426 highest predicting power (VE= 5.71%) followed by D*G interactive effect (VE= 5.09%) (Table
427 1.3, Sup. Table 1.5). **Glucose.** Diet was the strongest predictor of larvae glucose levels (VE=
428 17.8 %, p< 0.0001) (Table 1.3, Sup. Table 1.5). Other variables that produced a significant effect
429 on glucose levels were treatment (p= 0.0009), D*G (p = 0.05), and D*T (p= 0.0005) (Table 1.3,
430 Sup. Table 1.5).

431 **2) The symbiotic microbiota community composition of the larvae raised on the natural**
432 **diet was different from the lab food raised larvae and was influenced by maternally**
433 **inherited microbiota and the host's genotype.**

434 **2.1** The gut microbial community composition and diversity varied substantially across dietary
435 and treatment conditions. **Alpha diversity.** We characterized a total of 6,763 unique ZOTUs
436 across the whole dataset with the number of ZOTUs per sample ranging from 55 to 886. The
437 total number of reads per sample ranged from 4,685 to 908,308. Of the ZOTUs that could be
438 assigned a taxonomic classification, we identified 134 classes, 218 families, and 394 genera
439 represented across the samples. The response of alpha diversity to changing diets varied with the
440 larval sterilization treatment. For NS larvae, we found that the Shannon index of larvae raised on
441 PR and R diets was significantly higher than those raised on the PA diet (p= 0.0478 and p=
442 0.0252) (Sup. Table 2.1). All other comparisons were not significant. However, if the embryos
443 were subjected to sterilization, the microbial species richness of larvae raised on the PA diet was
444 significantly higher than larvae raised on the PR diet (p= 0.0371) (Sup. Table 2.1). In addition,
445 there was no significant difference in microbial species richness between larvae raised on regular
446 or peach regular diets (Sup. Table 2.1). We observed the exact same pattern for the PD whole
447 tree index. Larvae raised on any diet were not significantly different in Shannon's index (Sup.
448 Table 2.1).

449 **Beta diversity**. The hierarchical clustering of the Bray-Curtis distances indicated that the most
450 distant microbial communities were formed between larvae raised on the R and PR diet (Fig.
451 2A). This pattern held true for both sterilized and non-sterilized larvae (Fig. 2A). Clustering
452 Weighted Unifrac distances suggested that PR and R diets may produce symbiotic microbial
453 communities that were phylogenetically distant from each other, especially if the parental
454 microbiota had been removed (Fig. 2B).

455 **Taxa composition**. Applying discriminant analysis on the ten most abundant microorganisms at
456 each taxonomic level revealed which organisms were largely responsible for the differentiation

457 of the microbial composition on the canonical plot, based on diet. Thus, PR food is largely
458 defined by the abundance of Cyanobacteria at the phylum level (Sup. Fig. 1),
459 Epsilonproteobacteria at the class level (Sup. Fig. 2), Streptophyta at the order level (Sup. Fig.
460 3A, 3B), Leuconostocaceae sequences at the family level (Sup. Fig. 4), and *Leuconostoc* at the
461 genera level (Fig. 3A, Sup. Fig. 5). In turn, the lab diet was defined by Firmicutes, Bacilli,
462 Lactobacillales, Lactobacillaceae, and *Lactobacillus* respectively (Sup. Fig. 1-5, Fig. 3A).
463 Interestingly, when we considered only the 10 most abundant organisms at each taxonomic level,
464 we did not see a clear separation between R and PA diets unless the larvae were sterilized (Sup.
465 Fig. 1-5). If parental microbiota were removed, the differentiation of the PA diet was led by
466 Actinobacteria, Actinobacteria and Alphaproteobacteria, Clostridiales and Rickettsiales,
467 Lachnospiraceae and Rickettsiaceae, and *Bacteroides* and *Wolbachia* (Sup. Fig. 1B-5B, Fig. 3A).
468 Including all identified bacterial groups in the discriminant analysis revealed that diet was a good
469 predictor of bacterial taxa composition at phylum, class, order, family, genus, and even
470 individual ZOTU levels (Fig 3B, Sup. Fig.6-11).

471 Overall, for non-sterilized larvae the abundance of eight phyla, 12 classes, 20 orders, 27 families,
472 40 genera, and 141 ZOTUs were significantly different between PR and R food (Sup. Tables 3.1-
473 3.6). Comparing PR and PA food, we found that the abundance of four phyla, six classes, nine
474 orders, 16 families, 20 genera, and 76 ZOTUs were significantly different (Sup. Tables 3.1-3.6).
475 Lastly, we observed the significant difference for the abundance of four phyla, three classes, five
476 orders and families, three genera, and 27 ZOTUs between R and PA food (Sup. Tables 3.1-3.6).
477 This indicated the minimal difference between microbial communities of these diets to be
478 consistent with the discriminant analysis.

479 In larvae lacking parental microbiota, we observed that the abundance of six phyla, 10 classes,
480 18 orders, 34 families, 37 genera, and 114 ZOTUs were significantly different between lab and
481 peach diets (Sup. Tables 3.1-3.6). Comparing PR and PA diets, we found a significant difference
482 in abundance of seven phyla, 14 classes, 32 orders, 48 families, 67 genera, and 200 ZOTUs (Sup.
483 Tables 3.1-3.6). R and PA diets were significantly different in the abundance of five phyla, six
484 classes, nine orders, 13 families, 18 genera, and 87 ZOTUs (Sup. Tables 3.1-3.6).

485 **2.2** The maternally transmitted microbiota influenced the composition of the larvae's symbiotic
486 microbiota communities. **Alpha diversity.** S larvae had higher values for species richness,
487 Shannon, and PD whole tree indexes on the PA diet (Sup. Table 2.2). NS larvae had a
488 significantly higher Simpson index value on the PR diet (Sup. Table 2.2). All other comparisons
489 were not significantly different. **Beta diversity.** When comparing the difference between beta
490 diversity metrics in NS and S treatments for each diet, we observed a distinctive clustering,
491 based on the treatment of samples that were raised on PA food for Bray-Curtis Distance (Fig.
492 4A) and Weighted Unifrac distance (Fig. 4D). For the samples that were raised on R food, we
493 observed the clustering for Weighted Unifrac distance only (Fig 4F).

494 **Taxa composition.** The discriminant analysis indicated that the status of inheritance of the
495 parental microbiota could serve as a good predictor for differentiation of the bacterial community
496 as indicated with the canonical plot on all taxonomic levels (phylum, order, class, family, genus,
497 and ZOTU) (Fig.5 A-C, Sup. Fig. 12A-17C). Among the 10 most abundant phyla that defined
498 the differentiation of the NS community were Firmicutes and Bacterioidetes on the PA diet (Sup.
499 Fig. 18A), Firmicutes on the PR diet (Sup. Fig. 18B), and Proteobacteria, Tenericutes, and
500 Flusobacteria on the R diet (Sup. Fig. 18C). Phyla that were influential for differentiation of the
501 S community were Actinobacteria, Flusobacteria and Cyanobacteria on the PA diet (Sup. Fig.

502 18A), Actinobacteria, Tenericutes, and Flusobacteria on the PR food (Sup. Fig. 18B), and
503 Planctomycets and Bacterioides on the R diet (Sup. Fig. 18C). Considering bacterial classes, the
504 NS treatment was strongly defined by Bacilli on the PA (Sup. Fig. 19A) and the PR diets (Sup.
505 Fig. 19B), and Alphaproteobacteria and Bacteroida on the R food diet (Sup. Fig. 19C). The
506 sterilization treatment was mostly separated due to Actinobacteria on the PA diet, Alpha and
507 Beta proteobacteria on the PR diet, and Gammaproteobacteria on the R diet (Sup. Fig. 19A-C).
508 On R food the 95% confidence ellipses clearly collided, suggesting that S and NS microbial
509 communities might not be fully separated (Sup. Fig. 19C). At the order level, the NS community
510 was defined by Lactobcillales on the PA and the PR diets, and Actinomycetales and
511 Rhodospirillales on the R food (Sup. Fig. 20A-C). Sterilized larvae were associated with
512 abundances of Streptophyta on the PA food, as well as Burkholderiales and Rhodospirillales on
513 the PR diet (Sup. Fig. 20A-B). On the R diet, four out of ten tested orders were strongly
514 associated with the S treatment (Sup. Fig. 20C). At the family level, the NS larvae were
515 correlated with Lactobacillaceae on the PA and the PR diets and Acetobacteraceae on the R food
516 diet (Sup. Fig. 21A-C). Sterilized larvae were defined by the abundance of Nocardiaceae on the
517 PA food and Leuconostocaceae and Caulobactereceae on the R diet (Sup. Fig. 21A-C). On the
518 genera level, NS was primarily separated by *Lactobacillus* on the PA diet and *Acetobacter* and
519 *Agrobacterium* on the R food (Sup. Fig. 22A, C). On the PR food diet, 95% confidence ellipses
520 overlapped, indicating that sterilization status is a weak predictor for abundance of the 10 most
521 common genera (Fig. 5B). The S treatment was primarily defined by the abundance of
522 *Leuconostoc* and *Gluconobacter* on the PA diet and *Lactobacillus* and *Leuconostoc* on the R diet
523 (Fig. 5 A, C, Sup. Fig. 22A, C).

524 Multiple microbial groups were significantly different in their distribution between NS and S
525 treatments across the diets on all taxonomic levels. On regular food, there existed an abundance
526 of four phyla (Sup. Table 4.1), four classes (Sup. Table 4.2), five orders (Sup. Table 4.3), six
527 families (Sup. Table 4.4), nine genera (Sup. Table 4.5), and 41 ZOTUs (Sup. Table 4.6). On the
528 PR diet we saw a significant difference in the abundance of one phylum (Sup. Table 4.1), two
529 classes (Sup. Table 4.2), six orders (Sup. Table 4.3), nine families (Sup. Table 4.4), 11 genera
530 (Sup. Table 4.5), and 61 ZOTUs (Sup. Table 4.6). The highest number of significantly different
531 taxa was observed on the PA diet with seven phyla (Sup. Table 4.1), 12 classes (Sup. Table 4.2),
532 21 orders (Sup. Table 4.3), 34 families (Sup. Table 4.4), 43 genera (Sup. Table 4.5), and 148
533 ZOTUs (Sup. Table 4.6).

534 **2.3** The composition of the microbial community exhibited variation with host genotype, which
535 further exhibited a significant interactive effect with diet and treatment. We also tested the
536 influence of genotype and other variables' interactive effect on the abundance of microbiota. At
537 the phyla level, 14 were significantly influenced by genotype, three by D*G interaction, five by
538 G*T, five by D*T, and six by D*G*T (Sup. Table 5.1). Abundances of 30 classes were
539 significantly influenced by genotype, eight by D*G, G*T, and D*T, and 10 by D*G*T
540 interaction (Sup. Table 5.2). Among the orders, an abundance of 46 was significantly influenced
541 by genotype, 13 by D*G, 15 by G*T, 16 by D*T, and 15 by D*G*T (Sup. Table 5.3). The
542 abundance of 72 families was significantly influenced by genotype, 20 by D*G, 15 by G*T, 18
543 by D*T, and 23 by D*G*T (Sup. Table 5.4). Lastly, genotype significantly influenced 94 genera,
544 D*G influenced 44, G*T influenced 46, D*T influenced 30, and D*G*T influenced 45 genera
545 (Sup. Table 5.5).

546 **3.1** We identified microbial taxa that exhibited correlations with host phenotype across diets and
547 treatments, with many that had a diet, treatment or genotype specific relationship. Across the NS
548 larvae, we found four significant interactions on the R food, 14 on PR diet, and 14 on PA diet at
549 the phylum level (Sup. Table 6.1). For the S larvae at the same taxonomic level, we found eight
550 significant interactions on the R diet, five on the PR and two on PA food (Sup. Table 6.1). At the
551 class taxonomic level, nine significant interactions were found on R food, 35 on PR, and 24 on
552 PA diet (Sup. Table 6.2). Considering S larvae, there were 23 significant correlations on the R
553 diet, 11 on PR, and four on the PA diet (Sup. Table 6.2). For NS larvae at the order level, we
554 found 23 significant correlations on the R diet, 57 on PR, and 46 on PA diet (Sup. Table 6.3). For
555 S larvae we found 29 significant correlations on R food, 22 on PR, and nine on PA diet (Sup.
556 Table 6.3). At the family level, we found 34 significant correlations on R food, 88 on PR, and 67
557 on PA diet for NS larvae (Sup. Table 6.4). For S larvae, we observed 51 significant interactions
558 on R food, 29 on PR, and 14 on PA diets (Sup. Table 6.4). Across the genera, we found 40
559 significant interactions on R, 105 on PR, and 64 on PA diets, for NS larvae (Fig. 6-7, Sup. Table
560 6.5). Considering S larvae, we found 76 significant interactions between tested taxa and
561 phenotypes on R, 46 on PR, and 33 on PA diets (Fig. 6-7, Sup. Table 6.5). At the level of
562 individual ZOTUs, for NS larvae, we found 226 significant interactions on R, 283 on PR, and
563 225 on PA diets (Sup. Table 6.6). For S larvae the number of significant interactions between
564 ZOTUs abundances and larvae phenotypes were as follow 313 on R, 164 on PR, and 230 on PA
565 diets (Sup. Table 6.6).

566 We evaluated the interactive effect of the abundance of microbial taxa and diet, genotype, and
567 treatment on forming the tested phenotypes. D*A produced a significant effect in 11 cases at the
568 phylum level (Sup. Table 7.01), in 26 at the class level (Sup. Table 7.02), in 52 at the order level

569 (Sup. Table 7.03), in 72 at the family level (Sup. Table 7.04), and in 95 cases at the genus level
570 (Sup. Table 7.05). We found a significant G*A interaction in eight cases at the phylum level
571 (Sup. Table 7.06), in 10 at the class level (Sup. Table 7.07), in 39 at the order level (Sup. Table
572 7.08), in 57 at the family level (Sup. Table 7.09), and in 105 cases at the genus level (Sup. Table
573 7.10). T*A produced a significant effect in 13 cases at the phylum level (Sup. Table 7.11), in 27
574 at class level (Sup. Table 7.12), in 36 at the order level (Sup. Table 7.13), in 60 at the family
575 level (Sup. Table 7.14), and in 87 cases at the genus level (Sup. Table 7.15).

576 **Discussion**

577 **1.1: Overall, it appeared that frozen peach food was capable of providing nutritional**
578 **conditions similar to the natural ones and can preserve key microbial taxa necessary for**
579 **survival and development of *Drosophila* larvae.**

580 The reduction in survival, increase in development time and increase in triglyceride levels, as
581 well as decreased weight and protein levels of the larvae raised on the natural food compared
582 with the larvae raised on the R lab food resembles the phenotype generated by a reduced protein
583 diet. These findings correlate with our evaluation of the protein concentrations in different diets.
584 (Klepsatel et al., 2018, Bing et al., 2018, Skorupa et al., 2008, Sang, 1956). In addition, the
585 adaptation of *Drosophila* to the lab environment was connected to increased weight and reduced
586 stress tolerance (Sgro and Partridge, 2000, Hoffmann et al., 2001, Russell et al., 2012).
587 Therefore, nutritional and pathogenic stresses associated with the natural food conditions could
588 further contribute to the decrease in survival and development rate of larvae raised on the PR
589 food compared to the standard diet (Staubach et al., 2013, Bing et al., 2018, Pais et al., 2018,
590 Sang, 1956).

591 The pattern regarding glucose concentration was more interesting. Freshly unfrozen peach food
592 had a higher glucose concentration, but larvae raised on the PR diet had the lowest concentration
593 compared to larvae raised on any other diet. This pattern was likely caused by the activity of
594 naturally acquired microbes since it was shown that the presence of several microbial taxa that
595 naturally associate with *Drosophila,* such as *Acetobacter,* is correlated with decreased sugars in
596 fly food and *Drosophila* itself (Huang and Douglas, 2015, Dobson et al., 2015). In addition,
597 incubation of the PR food, even without the larvae, led to a drastic reduction of glucose levels
598 compared to the R and PA diets. Furthermore, the difference between all phenotypes (with the
599 exception of glucose) increased if the peach food diet was autoclaved and even more (with the
600 exception of triglyceride) if the parental microbiota were not transferred to the autoclaved diet.
601 This suggested that symbiotic microbiota drove the phenotypic change between PR and PA
602 raised larvae.

603 These findings are consistent with previous studies which showed that the presence of naturally
604 associated microbiota was advantageous for *Drosophila melanogaster* and *Drosophila suzukii* on
605 fresh fruit diets, which are also poor in protein content (Pais et al., 2018, Bing et al., 2018). In
606 fact, larvae raised on a PA diet closely resembled the phenotype of axenic larvae and axenic
607 larvae raised under low protein nutritional conditions. Examples of this similar phenotype
608 include lower survival and body size/weight (Shin et al., 2011, Wong et al., 2015, Dobson et al.,
609 2015), longer development time (Shin et al., 2011, Newell and Douglas, 2014a), elevated
610 glucose (Huang and Douglas, 2015) and triglyceride levels (Newell and Douglas, 2014a, Dobson
611 et al., 2015).

612 **1.2 Maternally deposited microbes produced positive effects on larvae that were raised on**
613 **the peach diets.**

614 Interestingly, the presence of parental microbiota did not produce a significant effect on any of
615 the tested phenotypes, when larvae were raised on the lab diet. Contrarily, on the peach diet, the
616 presence of parental microbota increased the weight and development rate even if the original
617 peach microbiota were still present. These findings are consistent with the reports of beneficial
618 effects, of the maternally deposited microbiota, for larvae on a fruit diet. These results also
619 indicate the importance of considering an organism's natural environmental conditions when
620 addressing the questions about symbiotic relationships and evolutionary patterns (Pais et al.,
621 2018, Bing et al., 2018).

622 **1.3 Genotype was one of the key factors that influenced larvae phenotypes.**

623 It is important to note that although the described patterns were observed for the total
624 experimental population of larvae, the genetic component still played a significant role in
625 generating all but the glucose phenotype. In addition, consistent with previous research, we
626 observed that D*G interaction played a significant role in forming metabolic phenotype as well
627 as contributed to the survival of the organism (Reed et al., 2010, Reed et al., 2014). Furthermore,
628 most of the tested phenotypes were significantly correlated with G*T and even D*G*T,
629 indicating the importance of considering multiple factors to understand the development of
630 complex traits.

631 **2.1 Microbiota of the larvae raised on PR food exhibit a distinct community structure and**
632 **might remind microbiota of wild flies.**

633 Multiple studies were performed to evaluate the gut microbiota composition of lab and wild
634 populations of *Drosophila* (Chandler et al., 2011, Adair et al., 2018, Douglas, 2018, Pais et al.,
635 2018, Wong et al., 2013). Although most of them consistently report the prevalence of different

636 members of Alphaproteobacteria, Bacilli, or Gammaproteobacteria in lab and wild populations,
637 the relative abundance of the taxa, especially at lower taxonomic levels, often varies between
638 studies (Douglas, 2018). In our work, larvae raised on the PR food diet formed a distinct
639 community clearly separated from the larvae raised on the R food diet, as displayed on the
640 canonical plot. We observed a higher prevalence of *Gluconobacter* and *Leuconostoc* and lower
641 abundance of *Lactobacillus* in larvae raised on the PR diet compared with the R food diet (Pais
642 et al., 2018, Staubach et al., 2013, Corby-Harris et al., 2007, Chandler et al., 2011), which is
643 consistent with previous findings performed on natural populations of *Drosophila.*

644 However, it is difficult to judge how well the microbial community of our experimental larvae
645 represent the microbial community of wild population, as the variety of factors could influence
646 gut microbiota composition in flies which certainly complicates the comparison between studies
647 (Douglas, 2018, Staubach et al., 2013, Jehrke et al., 2018). As such, it was shown that the gut
648 microbiota composition of lab reared flies may vary with diet (and even among the standard diets
649 with the major carbohydrate source), genetic line, development stage temperature, and etc.
650 (Jehrke et al., 2018, Douglas, 2018, Wong et al., 2011, Moghadam et al., 2018). The wild
651 populations of gut microbiota in *Drosophila* was shown to vary with collection location and diet
652 (Adair et al., 2018, Wong et al., 2013, Staubach et al., 2013, Martinez-Porchas et al., 2017). In
653 other insects and wild populations of vertebrates, gut microbiota was shown to change even with
654 seasonality (Behar et al., 2008, Ferguson et al., 2018, Tong and Zhang, 2019, Maurice et al.,
655 2015).

656 In addition, the relationship between *Drosophila* gut microbiota during the developmental and
657 adult stages is a subject of controversy between a few studies that compared those relationships
658 (Jehrke et al., 2018, Wong et al., 2011, Vacchini et al., 2017). Furthermore, to the best of our

659 knowledge, the gut microbiota of the larvae from the natural populations was not assessed at all.
660 This is likely due to the complexity of identifying *Drosophila* species during the larval stage.
661 Therefore, we hope to provide the methodology for the possibility of exploring the effects of a
662 natural diet, and the microbial community associated with it, in a controlled lab environment.
663 This setting provides the opportunity to work not only with adult flies but also with larvae.

664 **2.2-2.3 Community structure of symbiotic microbiota were correlated with diet, treatment,**
665 **host genotype and their specific interactive effects.**

666 The development of symbiotic microbiota populations was shown to be correlated with the
667 available nutrients present in the diet, the host's genotype and parental microbiota left on the
668 chorion of the egg (Douglas, 2018, Jehrke et al., 2018, Wong et al., 2015). Complementary to the
669 results reported by Jehkre (2018), we also observed that the genotype of the host may influence
670 the abundance of bacterial taxa more than the diet. Wong (2015) reported that the bacterial
671 population deposited on the *Drosophila* embryo may shift the symbiotic microbiota population
672 of the offspring, even in the presence of bacteria that previously colonized the food substrate. We
673 observed similar results in most cases.

674 However, among the 10 most abundant genera on the PR diet, the separation of the S and NS
675 larvae microbial community compositions was not present on the canonical plot indicating the
676 possibility of a difference in the response of the lab and the natural microbial population to the
677 presence of *Drosophila* parental microbiota. This differentiation was not likely caused by the
678 nutrition composition of the food since the PA separation, represented on the canonical plot,
679 between S and NS treatments was obvious in all cases. In addition, for beta diversity distances,
680 the abundance of individual microbial taxa, as well as the correlations between the abundances

681 of microbial taxa within the microbial community, represented patterns found in PA food that
682 resembled the ones in the R food raised larvae, if parental microbiota were not removed (Fig 4,
683 5, Sup. Fig.12-23). Overall, consistently with previous studies, our findings indicated the
684 dependency of relative microbial abundances on all of the tested variables and additionally on
685 the interactive effect between them (Wong et al., 2015, Jehrke et al., 2018, Douglas, 2018).

686 **3.1 The influence of individual microbial taxa as well as the influence of the whole**
687 **microbial community on the host may vary with the diet and other environmental and**
688 **genetic conditions.**

689 Genotype and gut microbiota composition are among the major factors that control the
690 development of obesity traits (Parks et al., 2013). Changes in some key microbiota populations
691 are associated with the rapid expansion in the prevalence of metabolic syndrome (Zhang et al.,
692 2010). Alterations in the gut microbiota community can modulate insulin secretion and
693 sensitivity, thus contributing to diabetes susceptibility (Kreznar et al., 2017). Moreover, previous
694 research indicates that genetic variation considerably influences the gut microbiota composition
695 (Zhang et al., 2010, Kreznar et al., 2017, Jehrke et al., 2018). However, most of the studies
696 mentioned above have used less than ten genotypes to study the correlation between gut
697 microbiota and the pathogenesis of obesity in mice. The challenges of using a mouse model
698 involve relatively high expenses for husbandry and logistics (Rosenthal and Brown, 2007, Berger
699 et al., 2005, Martin et al., 2016).
700 *Drosophila melanogaster* is an exceptional model to study the effect of genotype on the
701 phenotype formation, due to the variety of established tools such as Drosophila Genetic
702 Reference Panel and The Drosophila Synthetic Population Resource. These resources offer a
703 variety of diverse genotypes, with sequenced parental genomes, that allow for testing the

704 microbiota effects across various genetic backgrounds and provides potential for studying
705 genetic interaction between host and its symbionts, and even mapping the specific genetic loci
706 responsible for the interactions (Mackay et al., 2012, King et al., 2012, Chaston et al., 2016). The
707 phenotypic response to a diet modification often varies with the genotype (Reed et al., 2010,
708 Reed et al., 2014). In fact, diet by genotype (DxG) interaction may explain more variance than
709 diet alone in the metabolic response of such traits, such as triglyceride and carbohydrate
710 concentrations (Reed et al., 2010). In addition, recent findings showed that genotype by diet
711 interactions significantly influences metabolomic profiles; hence, laying the foundation for
712 explaining the mechanism through which DxG influences metabolic traits (Reed et al., 2014,
713 Williams et al., 2015). Our results are consistent with previous research, in that phenotypic
714 response varied significantly between genetic lines (Reed et al., 2014, Williams et al., 2015).
715 Genotype had a significant effect on survival, development rate, and triglyceride levels, and was
716 the second-best predictor of weight and the best predictor of protein levels.
717 Obesity and type two diabetes are associated with elevated weight, high blood glucose
718 concentrations, and excess accumulation of adipose tissue (Martyn et al., 2008, Akter et al.,
719 2017). Consistent with recent studies linking *Lactobacillus* and *Coprococcus* to obesity in
720 humans, our results show that these genera are positively associated with glucose levels (Million
721 et al., 2012, Ignacio et al., 2016, Murugesan et al., 2015, Armougom et al., 2009). In addition,
722 previous research has shown an overall decrease in the abundance of *Firmicutes* in obese humans
723 (Schwiertz et al., 2010). Similarly, we observed that the total abundance of *Firmicutes* is
724 negatively associated with triglyceride levels. It should be noted that the correlation between
725 metabolic phenotype and particular microbial taxa could vary between studies (Ley et al., 2006,
726 Furet et al., 2010, Turnbaugh et al., 2006).

727 Consistently with previous *Drosophila* research, we observed that the abundance of microbial
728 taxa was correlated with measured phenotypes. Such as *Acetobacteraceae* is negatively
729 correlated with larvae glucose levels (Chaston et al., 2016, Douglas, 2018, Chaston et al., 2014).
730 Additionally, *Acetobacter* increases development time while *Lactobacillus* and *Firmicutes*
731 decreases it (Chaston et al., 2014, Chaston et al., 2016, Newell and Douglas, 2014a, Storelli et
732 al., 2011). Previous work showed that *Acetobacter* species reduced triglyceride levels while most
733 *Lactobacillus* species had no effect (Chaston et al., 2016, Newell and Douglas, 2014a). In
734 contrast, our data shows that *Acetobacter* did not significantly affect triglyceride levels, and
735 *Lactobacillus* showed a negative correlation. Consistent with Newell and Douglas (2014b), we
736 found that *L. brevis* and *L. plantarum* had no significant effect on protein levels, but in addition,
737 our results indicated that the abundance *Acetobacter* was negatively correlated with the protein
738 levels.

739 Consistently with Jehrke (2018), we observed that most of the correlations between the tested
740 phenotype and abundance of microbiota are relatively weak. Weaker correlations observed with
741 large sample sizes in microbiome research, while significant, fail to hold up to the use of stricter
742 FDR values or other conservative p adjustment methods (Jehrke et al., 2018, Wu et al., 2011).
743 Expanding the analysis of the bacterial species abundance for each phenotype beyond the most
744 dominant species, while providing a more complete overview of the correlation between tested
745 phenotypes and microbial abundance, also raises FDR values as a result of increasing sample
746 sizes (Wu et al., 2011). Previous microbiome studies have dealt with high FDR values by
747 accepting higher thresholds, so as to not miss possible correlations (Wu et al., 2011). Since the
748 level of FDR that should be tolerated is poorly defined and often widely variable compared to
749 accepted p-values, its value is often seen as arguable (Pawitan et al., 2005). Considering the large

750 sample sizes used in our analysis, using a low FDR value may obscure important correlations
751 between the tested phenotypes and their abundance of microbiota (Wu et al., 2011).

752 Some of the inconsistencies between our work and previous studies on the correlation of the
753 abundance of microbial taxa and measured metabolic phenotype, perhaps may be addressed to
754 the interactive effect between the variables included in the experiments. Several studies showed
755 that the contribution of the symbiotic microbiota to the host may be observed only in a diet
756 dependent manner. Such as Shin et al. (2011), showed that axenic *Drosophila* larvae would not
757 be able to develop on a protein poor diet without activation of the insulin signaling pathway by
758 its symbiotic microbe. Wong et al. (2014) found diet-dependent differences in microbiota
759 produced effect, including differences in vitamin microbial sparing on a low-yeast diet and
760 suppression of lipid and carbohydrate storage on a high-sugar diet. Bing et. al (2018) found that
761 symbiotic microbiota of *D. suzukii* are critical for providing proteins for development of flies
762 raised on fresh fruit, but that these microbial proteins are not essential for development of flies
763 raised on a nutrient sufficient diet.

764 In our study, we also observed that the effect that microbial abundance at the level of individual
765 taxa produced on larvae phenotype varied with the diet. In few cases even the direction of the
766 correlation between the abundance of microbial taxa and tested phenotype was opposite on
767 different diets. In addition, using PCA, we observed that correlational effects that microbial
768 abundance (as an example at the family level) produced on measured metabolic (Sup. Fig. 24A)
769 and fitness phenotypes (development rate and survival) (Sup. Fig 24B) varied between the diets.
770 The correlation coefficients for the influence of all microbial taxa on measured metabolic
771 phenotypes clustered together for the PR diet but not for other diets (Sup. Fig 24A). The
772 correlation coefficients between microbiota abundances and fitness phenotypes clustered for all

773 but PA diets (Sup. Fig 24B). Taking into account all of the described findings, perhaps in future
774 studies that will aim to understand the mechanisms for formation of metabolic phenotypes, we
775 will see a consistent control for the gut microbiota composition in a similar fashion as now we
776 can see it for genetic lines and nutritional composition of the food.

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Figures

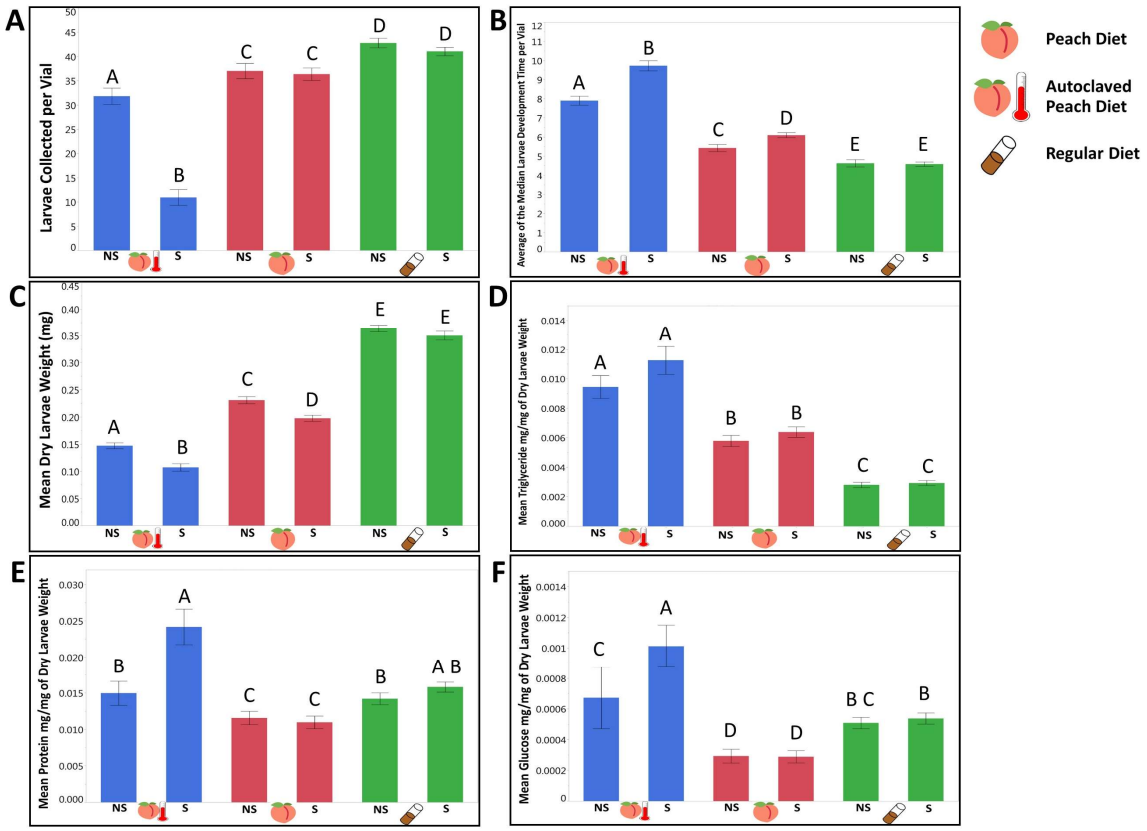

Figure 1: The Influence of diet and treatment on larvae mean A) survival until late 3rd instar stage B) development time C) weight D) Triglyceride E) Protein F) Glucose per mg of dry larvae weigh. Error bars indicated standard error.

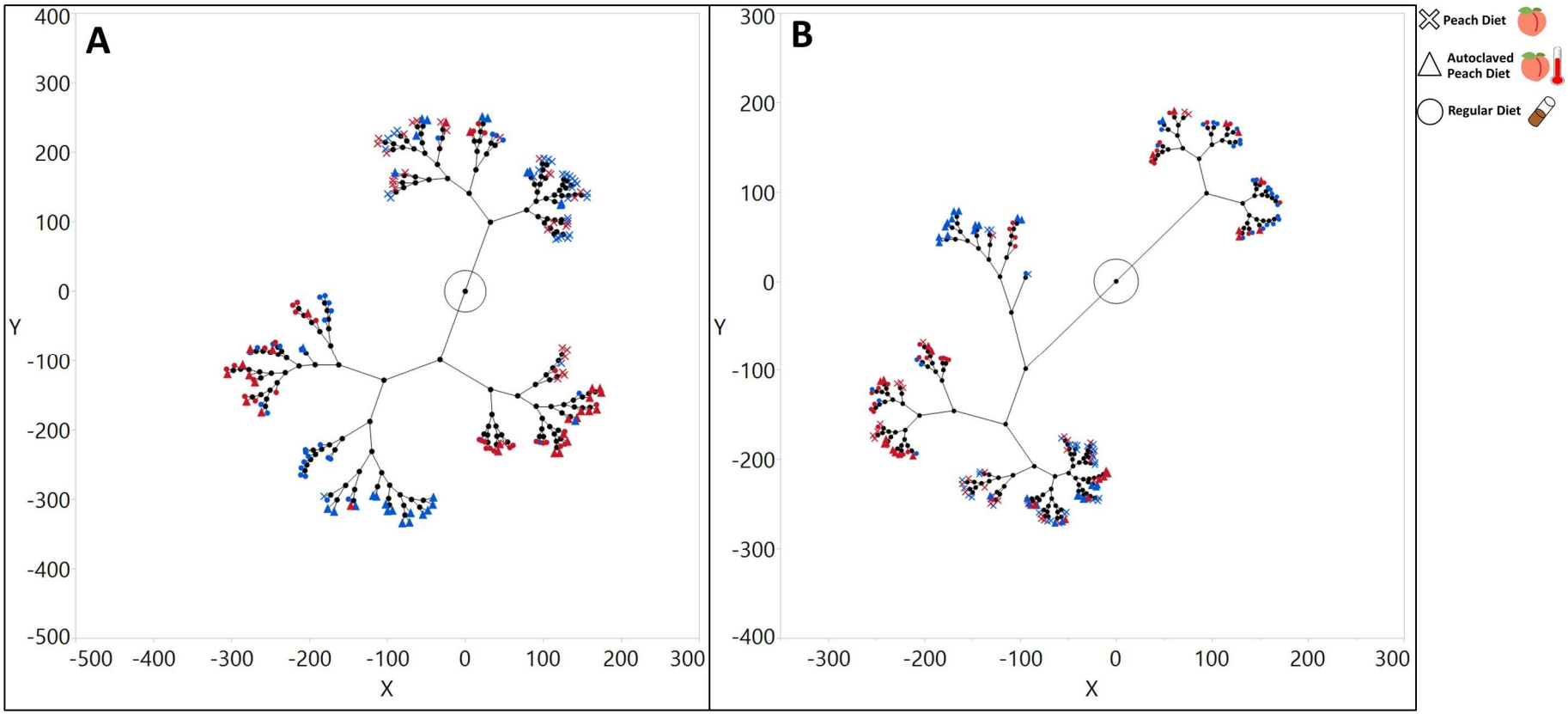

Figure 2: The influence of the diet on beta diversity distances between larvae symbiotic microbiota communities. Constellation plot based on hierarchal clustering of larvae microbiota community beta diversity distances A) Bray-Curtis distances B) Weighed Unifrac distances. Samples from sterilized larvae are marked with a blue color and samples from non-sterilized larvae are marked with the red color. Samples raised on regular lab diet are marked with circled, on a peach diet with crosses and on peach autoclaved diet with triangles.

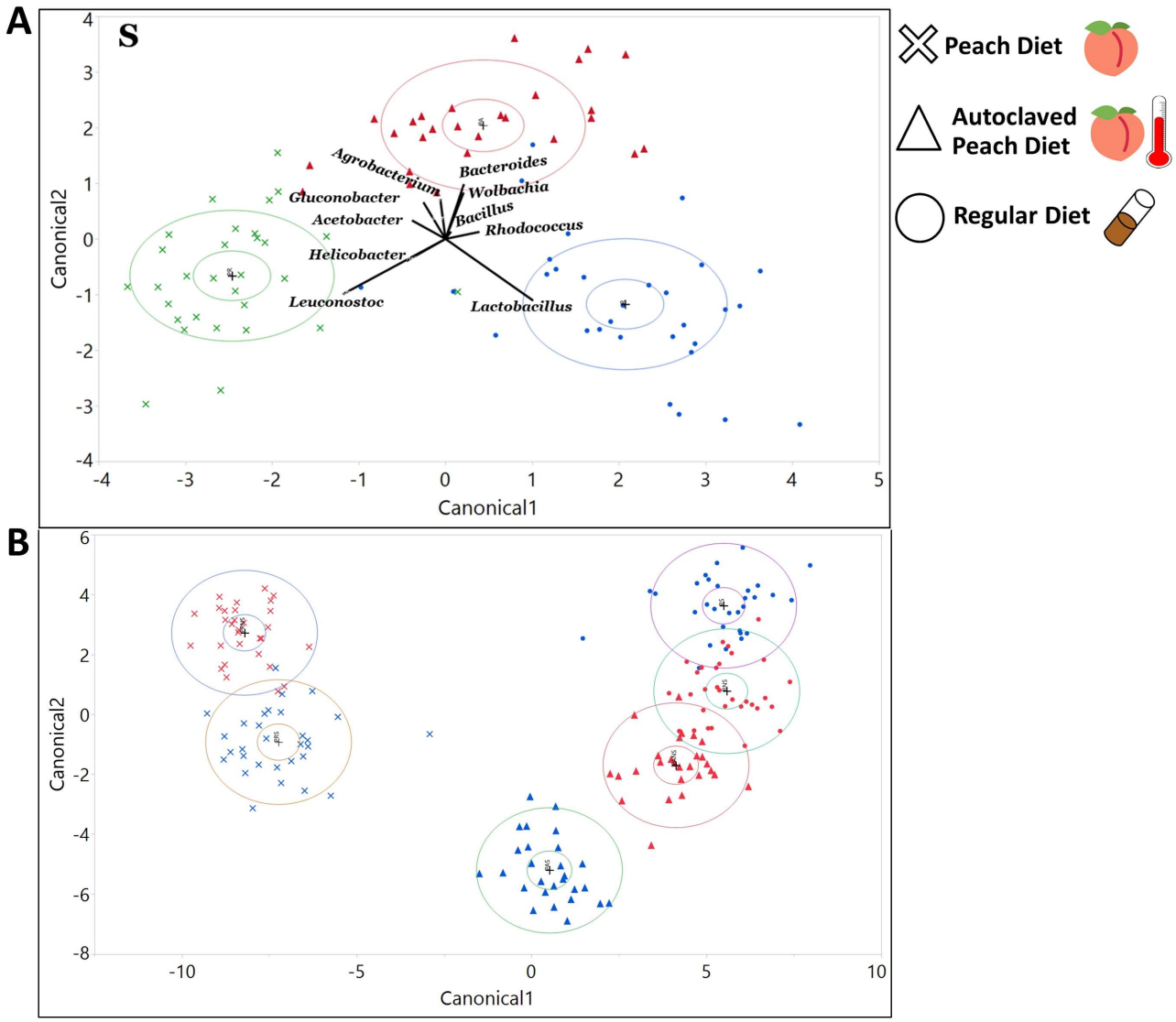

Figure 3: The influence of the diet on larvae symbiotic microbiota communities' composition. Discriminant analysis of symbiotic microbiota community based on taxa relative abundances in larvae from non-sterilized (red) and sterilized (blue) treatments. A) 10 most abundant microbial genera found in sterilized larvae. The length of the vector is correlated with the strength of the impact that it produced for the samples to be separated, in the vector direction, on the canonical plot B) 100 most abundant microbial zotu found in both sterilized and non-sterilized larvae. Non sterilized samples are marked with red color and sterilized larvae are marked with blue color.

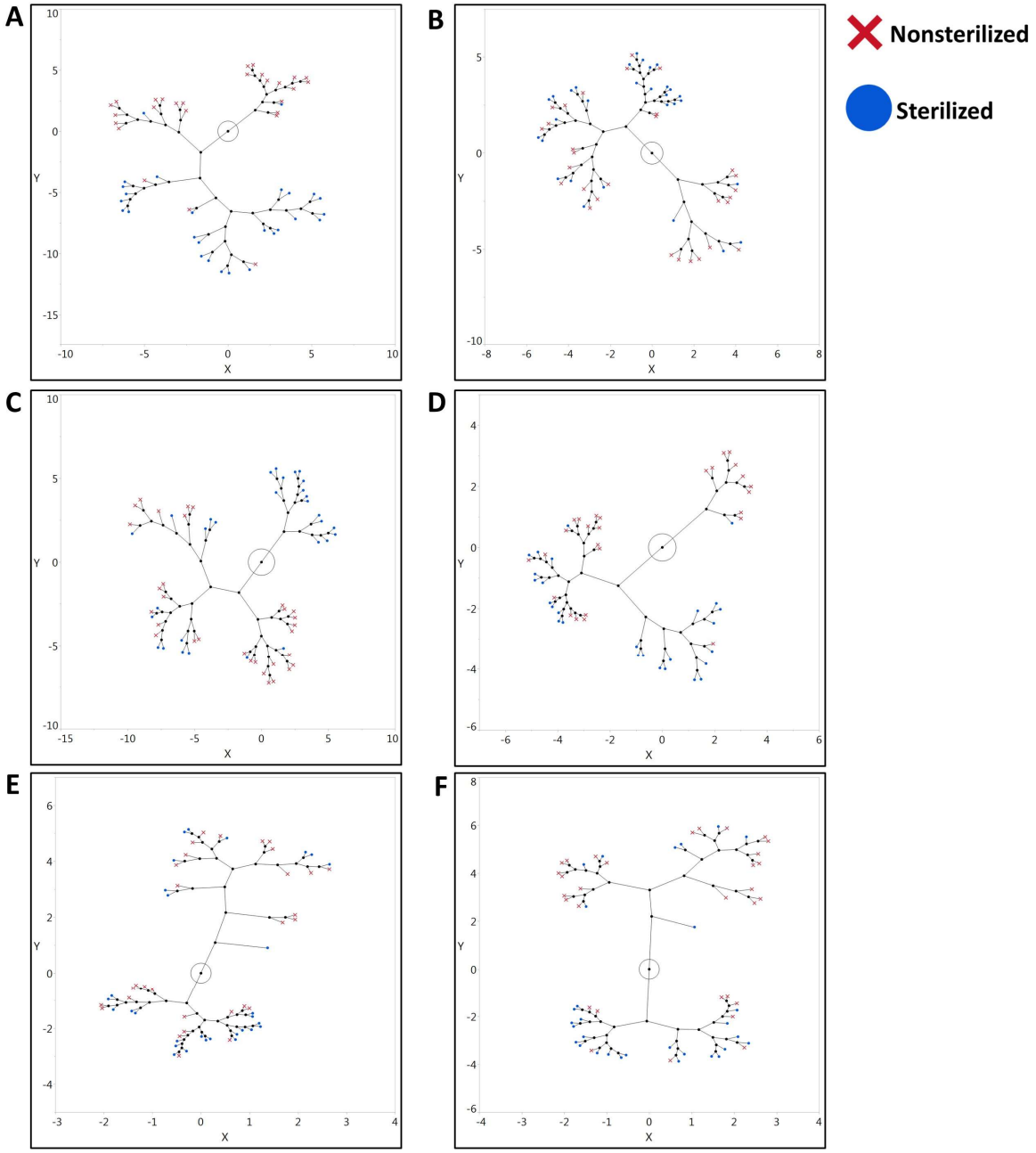

Figure 4: The influence of the treatment on beta diversity distances between larvae symbiotic microbiota communities. Hierarchal clustering of larvae microbiota community beta diversity distances A-C) Bray-Curtis distances between NS and S larvae raised on PA, PR, and R diets, respectively D-F) Weighed Unifrac distances between NS and S larvae raised on PA, PR, and R diets, respectively.

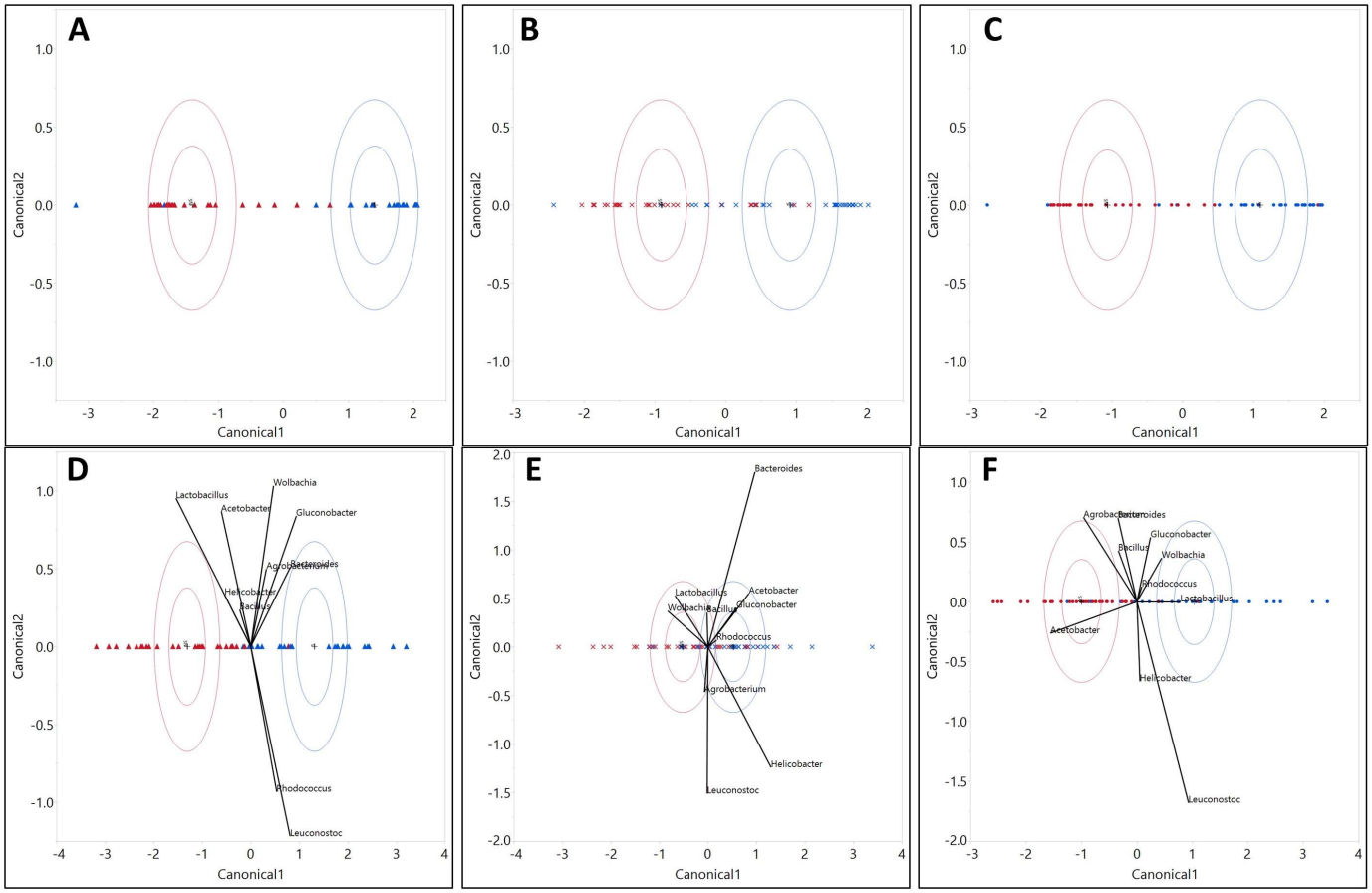

Figure 5: The influence of the treatment on larvae symbiotic microbiota communities' composition. Discriminant analysis of symbiotic microbiota community based on taxa relative abundances in larvae from non-sterilized (red) and sterilized (blue) treatments. The analysis includes A) All identified ZOTUs on a PA diet B) All identified ZOTUs on a PR diet C) All identified ZOTUs on a R diet D) 10 dominant genera on a PA diet E) 10 dominant genera on a PR diet F) 10 dominant genera on a R diet. In figures D-F, the length of the vector is correlated with the strength of the impact that it produced for the samples to be separated in the vector direction, on a canonical plot.

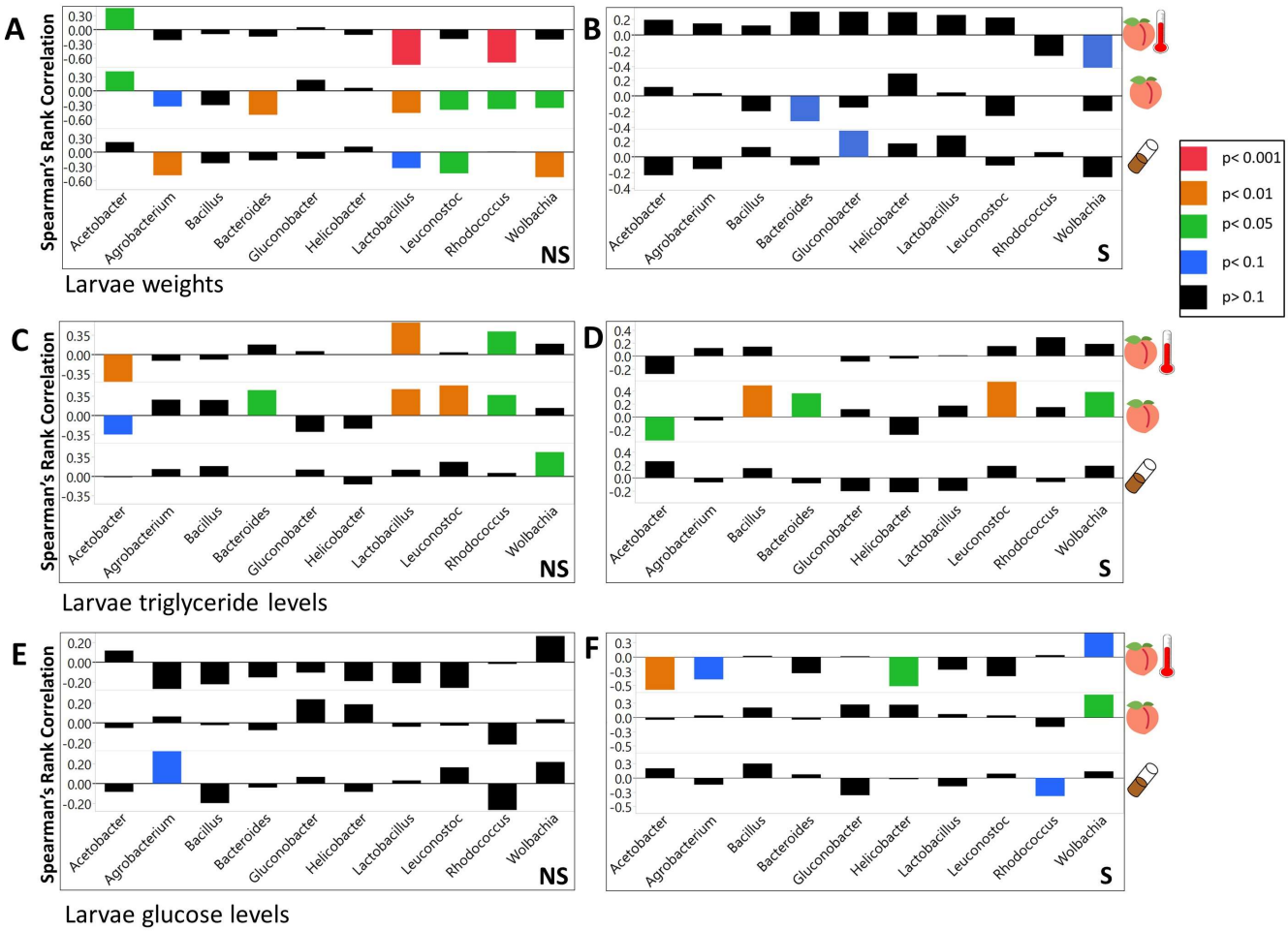

Figure 6: The influence of symbiotic microbial taxa on larvae metabolic phenotypes. Spearman's rank correlation coefficients between the abundance of 10 dominant symbiotic microbiota genera and larvae phenotypes, on each diet. The color of the bars corresponds to the level of significance for each correlation. A) Weight of NS larvae B) Weight of S larvae C) Triglyceride levels of NS larvae D) Triglyceride levels of S larvae E) Glucose levels of NS larvae F) Glucose levels of S larvae

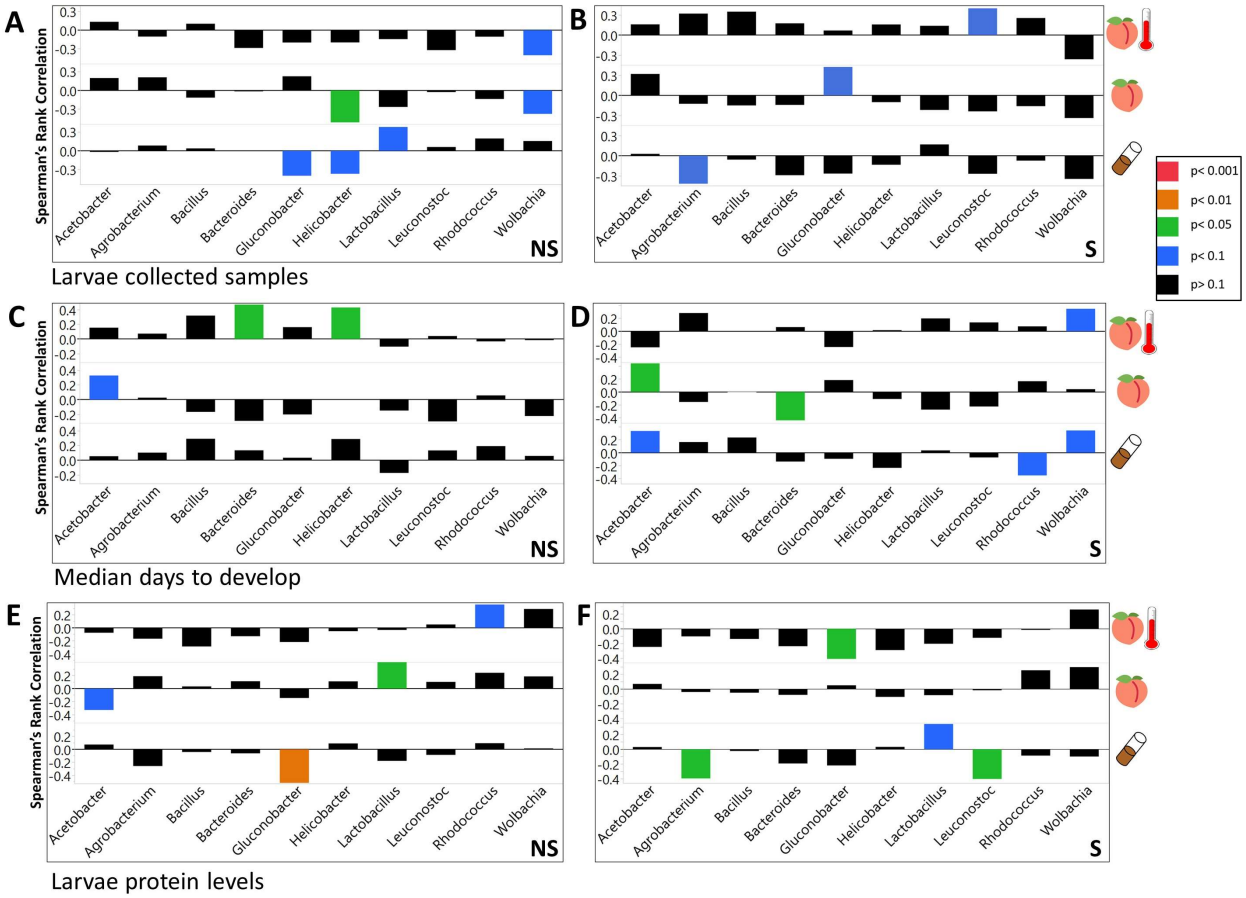

Figure 7: The influence of symbiotic microbial taxa on larvae life history traits and metabolic phenotypes. Spearman's rank correlation coefficients between the abundance of 10 dominant symbiotic microbiota genera and larvae phenotypes on each diet. The color of the bars corresponds to the level of significance for each correlation. A) Total number of collected NS larvae B) Total number of collected S larvae C) Median number of days to reach pre-pupation stage for NS larvae D) Median number of days to reach pre-pupation stage for S larvae E) Protein levels of NS larvae F) Protein levels of S larvae

Data Tables

Treatment	Survival	Development	Weight	Triglyceride	Protein	Glucose
NS	R> PR **	R< PR ***	R> RP ***	R< PR ***	R> PR **	R> PR ***
S	R> PR **	R< PR ***	R> PR ***	R< PR ***	R> PR ***	R> PR ***
NS	PR> PA ***	PR< PA ***	PR> PA ***	PR< PA ***	PR< PA	PR< PA **
S	PR> PA ***	PR< PA ***	PR> PA ***	PR< PA ***	PR< PA ***	PR< PA ***

Table 1.1: The influence of diet on larvae phenotypes. Comparison of larvae life history traits and metabolic phenotypes between larvae, from all 10 genetic lines, raised on regular lab diet (R), peach diet (PR), and autoclaved peach diet (PA). NS stands for non-sterilized larvae, S stands for sterilized larvae. Asterisks indicate the significance of comparisons $p< 0.001$ ***, $p< 0.01$ **, and $p< 0.5$ *

Genetic line	Diet	Survival	Development	Weight	Triglyceride	Protein	Glucose
All	PA	S< NS ***	S> NS ***	S< NS ***	S> NS	S> NS **	S> NS **
All	PR	S< NS	S> NS ***	S< NS ***	S> Ns	S< NS	S> NS
All	R	S< NS	S> NS	S< NS	S> NS	S> NS	S> NS

Table 1.2: The influence of parental microbiota on larvae phenotypes. Comparison of larvae life history traits and metabolic phenotypes between larvae, from all 10 genetic lines, raised on regular lab diet (R), peach diet (PR), and autoclaved peach diet (PA). NS stands for non-sterilized larvae, S stands for sterilized larvae. Asterisks indicate the significance of comparisons $p< 0.001$ ***, $p< 0.01$ **, and $p< 0.5$ *

Independent variable	Survival	Development	Weight	Triglyceride	Protein	Glucose
Diet	VE=28.4% ***	VE= 47.5% ***	VE= 31.4% ***	VE= 41.4% ***	VE= 4.24% ***	VE= 17.8% ***
Genetic line	VE=5.13% ***	VE=4.98% ***	VE=7.44% ***	VE=2.36% **	VE=5.71% ***	VE=3.15%
Treatment	VE=4.69% ***	VE=2.41% ***	VE=0.74% ***	VE= 0.36%	VE=0.40% *	VE= 2.61% ***
Diet*Genetic line	VE=3.13% ***	VE=0.96%	VE=2.88% ***	VE=5.03% ***	VE=5.09% ***	VE=5.52%
Diet*Treatment	VE=8.02% ***	VE=1.41% ***	VE=0.10%	VE=0.07%	VE=0.45%	VE= 3.55% ***
Genetic line*Treatment	VE=0.98% *	VE=2.82% ***	VE=1.06% ***	VE= 1.81% *	VE=3.53% ***	VE=0.72%
Diet*Treatment*Genetic line	VE= 2.37% ***	VE=0.82%	VE=2.75% ***	VE=4.81% ***	VE= 4.92% ***	VE= 2.37%

Table 1.3: The contribution of diet, genotype, treatment, and their interactive effects on formation of larvae life history traits and metabolic phenotypes. VE stands for variance explained, by each independent variable. Asterisks indicate the significance of comparisons $p< 0.001$ ***, $p< 0.01$ **, and $p< 0.5$ *

References

ADAIR, K. L., WILSON, M., BOST, A. & DOUGLAS, A. E. 2018. Microbial community assembly in wild populations of the fruit fly Drosophila melanogaster. *The ISME Journal*, 1.

AKTER, R., NESSA, A., HUSAIN, M., WAHED, F., KHATUN, N., YESMIN, M., NASREEN, S. & TAJKIA, T. J. M. M. J. M. 2017. Effect of Obesity on Fasting Blood Sugar. 26, 7-11.

ARMOUGOM, F., HENRY, M., VIALETTES, B., RACCAH, D. & RAOULT, D. J. P. O. 2009. Monitoring bacterial community of human gut microbiota reveals an increase in Lactobacillus in obese patients and Methanogens in anorexic patients. 4.

ASHBURNER, M. 1989. *Drosophila. A laboratory handbook*, Cold Spring Harbor Laboratory Press.

BEHAR, A., JURKEVITCH, E. & YUVAL, B. 2008. Bringing back the fruit into fruit fly–bacteria interactions. *Molecular Ecology,* 17, 1375-1386.

BERGER, Z., TTOFI, E. K., MICHEL, C. H., PASCO, M. Y., TENANT, S., RUBINSZTEIN, D. C. & O'KANE, C. J. J. H. M. G. 2005. Lithium rescues toxicity of aggregate-prone proteins in Drosophila by perturbing Wnt pathway. 14, 3003-3011.

BING, X., GERLACH, J., LOEB, G. & BUCHON, N. J. M. 2018. Nutrient-dependent impact of microbes on Drosophila suzukii development. 9, e02199-17.

BIRSE, R. T., CHOI, J., REARDON, K., RODRIGUEZ, J., GRAHAM, S., DIOP, S., OCORR, K., BODMER, R. & OLDHAM, S. 2010. High-fat-diet-induced obesity and heart dysfunction are regulated by the TOR pathway in Drosophila. *Cell metabolism,* 12, 533-544.

BOLGER, A. M., LOHSE, M. & USADEL, B. J. B. 2014. Trimmomatic: a flexible trimmer for Illumina sequence data. 30, 2114-2120.

BORDENSTEIN, S. R. & THEIS, K. R. 2015. Host Biology in Light of the Microbiome: Ten Principles of Holobionts and Hologenomes. *Plos Biology,* 13.

BRADFORD, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical biochemistry,* 72, 248-254.

BRUCE-KELLER, A. J., SALBAUM, J. M., LUO, M., BLANCHARD IV, E., TAYLOR, C. M., WELSH, D. A. & BERTHOUD, H.-R. J. B. P. 2015. Obese-type gut microbiota induce neurobehavioral changes in the absence of obesity. 77, 607-615.

BRUNO, D., BONELLI, M., DE FILIPPIS, F., DI LELIO, I., TETTAMANTI, G., CASARTELLI, M., ERCOLINI, D. & CACCIA, S. J. A. E. M. 2019. The intestinal microbiota of Hermetia illucens larvae is affected by diet and shows a diverse composition in the different midgut regions. 85, e01864-18.

CAPORASO, J. G., BITTINGER, K., BUSHMAN, F. D., DESANTIS, T. Z., ANDERSEN, G. L. & KNIGHT, R. J. B. 2010a. PyNAST: a flexible tool for aligning sequences to a template alignment. 26, 266-267.

CAPORASO, J. G., KUCZYNSKI, J., STOMBAUGH, J., BITTINGER, K., BUSHMAN, F. D., COSTELLO, E. K., FIERER, N., PENA, A. G., GOODRICH, J. K. & GORDON, J. I. J. N. M. 2010b. QIIME allows analysis of high-throughput community sequencing data. 7, 335.

CARVALHO, G. B., KAPAHI, P. & BENZER, S. 2005. Compensatory ingestion upon dietary restriction in Drosophila melanogaster. *Nature methods,* 2, 813.

CHANDLER, J. A., LANG, J. M., BHATNAGAR, S., EISEN, J. A. & KOPP, A. 2011. Bacterial communities of diverse Drosophila species: ecological context of a host–microbe model system. *PLoS genetics,* 7, e1002272.

CHASTON, J. M., DOBSON, A. J., NEWELL, P. D. & DOUGLAS, A. E. 2016. Host genetic control of the microbiota mediates the Drosophila nutritional phenotype. *Applied and environmental microbiology,* 82, 671-679.

CHASTON, J. M., NEWELL, P. D. & DOUGLAS, A. E. J. M. 2014. Metagenome-wide association of microbial determinants of host phenotype in Drosophila melanogaster. 5**,** e01631-14.
CLARK, A. & KEITH, L. J. G. 1988. Variation among extracted lines of Drosophila melanogaster in triacylglycerol and carbohydrate storage. 119**,** 595-607.
CORBY-HARRIS, V., PONTAROLI, A. C., SHIMKETS, L. J., BENNETZEN, J. L., HABEL, K. E. & PROMISLOW, D. E. 2007. Geographical distribution and diversity of bacteria associated with natural populations of Drosophila melanogaster. *Applied and environmental microbiology,* 73**,** 3470-3479.
DE LUCA, M., YI, N., ALLISON, D. B., LEIPS, J. & RUDEN, D. M. J. O. R. 2005. Mapping quantitative trait loci affecting variation in Drosophila triacylglycerol storage. 13**,** 1596-1605.
DEW-BUDD, K., JARNIGAN, J. & REED, L. K. 2016. Genetic and sex-specific transgenerational effects of a high fat diet in Drosophila melanogaster. *PloS one,* 11**,** e0160857.
DOBSON, A. J., CHASTON, J. M., NEWELL, P. D., DONAHUE, L., HERMANN, S. L., SANNINO, D. R., WESTMILLER, S., WONG, A. C.-N., CLARK, A. G. & LAZZARO, B. P. 2015. Host genetic determinants of microbiota-dependent nutrition revealed by genome-wide analysis of Drosophila melanogaster. *Nature communications,* 6**,** 6312.
DOUGLAS, A. E. J. L. A. 2018. The Drosophila model for microbiome research. 47**,** 157-164.
EARLY, A. M., SHANMUGARAJAH, N., BUCHON, N. & CLARK, A. G. 2017. Drosophila Genotype Influences Commensal Bacterial Levels. *PloS one,* 12**,** e0170332.
EDGAR, R. C. J. B. 2010. Search and clustering orders of magnitude faster than BLAST. 26**,** 2460-2461.
FERGUSON, L. V., DHAKAL, P., LEBENZON, J. E., HEINRICHS, D. E., BUCKING, C. & SINCLAIR, B. J. J. F. E. 2018. Seasonal shifts in the insect gut microbiome are concurrent with changes in cold tolerance and immunity. 32**,** 2357-2368.
FLINT, H. J., DUNCAN, S. H. & LOUIS, P. 2017. The impact of nutrition on intestinal bacterial communities. *Current Opinion in Microbiology,* 38**,** 59-65.
FURET, J.-P., KONG, L.-C., TAP, J., POITOU, C., BASDEVANT, A., BOUILLOT, J.-L., MARIAT, D., CORTHIER, G., DORÉ, J. & HENEGAR, C. 2010. Differential adaptation of human gut microbiota to bariatric surgery–induced weight loss. *Diabetes,* 59**,** 3049-3057.
HAN, T. S. & LEAN, M. E. 2016. A clinical perspective of obesity, metabolic syndrome and cardiovascular disease. *JRSM cardiovascular disease,* 5**,** 2048004016633371.
HOFFMANN, A. A., HALLAS, R., SINCLAIR, C. & PARTRIDGE, L. J. E. 2001. Rapid loss of stress resistance in Drosophila melanogaster under adaptation to laboratory culture. 55**,** 436-438.
HUANG, J.-H. & DOUGLAS, A. E. 2015. Consumption of dietary sugar by gut bacteria determines Drosophila lipid content. *Biology letters,* 11**,** 20150469.
HUANG, W., MASSOURAS, A., INOUE, Y., PEIFFER, J., RÀMIA, M., TARONE, A. M., TURLAPATI, L., ZICHNER, T., ZHU, D. & LYMAN, R. F. 2014. Natural variation in genome architecture among 205 Drosophila melanogaster Genetic Reference Panel lines. *Genome research,* 24**,** 1193-1208.
IGNACIO, A., FERNANDES, M. R., RODRIGUES, V. A., GROPPO, F. C., CARDOSO, A. L., AVILA-CAMPOS, M., NAKANO, V. J. C. M. & INFECTION 2016. Correlation between body mass index and faecal microbiota from children. 22**,** 258. e1-258. e8.
JEHRKE, L., STEWART, F. A., DROSTE, A. & BELLER, M. J. S. R. 2018. The impact of genome variation and diet on the metabolic phenotype and microbiome composition of Drosophila melanogaster. 8**,** 1-15.
KING, E. G., MACDONALD, S. J. & LONG, A. D. 2012. Properties and power of the Drosophila Synthetic Population Resource for the routine dissection of complex traits. *Genetics,* 191**,** 935-949.

KLEPSATEL, P., PROCHÁZKA, E. & GÁLIKOVÁ, M. J. E. G. 2018. Crowding of Drosophila larvae affects lifespan and other life-history traits via reduced availability of dietary yeast. 110**,** 298-308.

KREZNAR, J. H., KELLER, M. P., TRAEGER, L. L., RABAGLIA, M. E., SCHUELER, K. L., STAPLETON, D. S., ZHAO, W., VIVAS, E. I., YANDELL, B. S. & BROMAN, A. T. J. C. R. 2017. Host genotype and gut microbiome modulate insulin secretion and diet-induced metabolic phenotypes. 18**,** 1739-1750.

KUMAR, R., EIPERS, P., LITTLE, R. B., CROWLEY, M., CROSSMAN, D. K., LEFKOWITZ, E. J. & MORROW, C. D. 2014. Getting started with microbiome analysis: sample acquisition to bioinformatics. *Current protocols in human genetics***,** 18.8. 1-18.8. 29.

LEBOFFE, M. J. & PIERCE, B. E. 2012. *Microbiology: laboratory theory and application*, Morton Publishing Company.

LEITÃO-GONÇALVES, R., CARVALHO-SANTOS, Z., FRANCISCO, A. P., FIOREZE, G. T., ANJOS, M., BALTAZAR, C., ELIAS, A. P., ITSKOV, P. M., PIPER, M. D. & RIBEIRO, C. 2017. Commensal bacteria and essential amino acids control food choice behavior and reproduction. *PLoS biology,* 15**,** e2000862.

LEY, R. E., TURNBAUGH, P. J., KLEIN, S. & GORDON, J. I. J. N. 2006. Human gut microbes associated with obesity. 444**,** 1022-1023.

MACKAY, T. F., RICHARDS, S., STONE, E. A., BARBADILLA, A., AYROLES, J. F., ZHU, D., CASILLAS, S., HAN, Y., MAGWIRE, M. M. & CRIDLAND, J. M. 2012. The Drosophila melanogaster genetic reference panel. *Nature,* 482**,** 173.

MARTIN, T. L., BALSER, S. R., YOUNG, G. S. & LEWIS, S. D. J. J. O. T. A. A. F. L. A. S. 2016. Cost and effectiveness of commercially available nesting substrates for Deer Mice (Peromyscus maniculatus). 55**,** 412-418.

MARTINEZ-PORCHAS, M., VILLALPANDO-CANCHOLA, E., SUAREZ, L. E. O. & VARGAS-ALBORES, F. 2017. How conserved are the conserved 16S-rRNA regions? *PeerJ,* 5**,** e3036.

MARTYN, J. J., KANEKI, M. & YASUHARA, S. J. A. 2008. Obesity-induced insulin resistance and hyperglycemia: etiological factors and molecular mechanisms. 109**,** 137.

MATURIN, L., PEELER, J. J. B. A. M. U. F. & DRUG ADMINISTRATION, N. H. A. S. S. U. 2001. BAM aerobic plate count.

MAURICE, C. F., KNOWLES, S. C., LADAU, J., POLLARD, K. S., FENTON, A., PEDERSEN, A. B. & TURNBAUGH, P. J. J. T. I. J. 2015. Marked seasonal variation in the wild mouse gut microbiota. 9**,** 2423-2434.

MENDEZ, S., WATANABE, L., HILL, R., OWENS, M., MORACZEWSKI, J., ROWE, G. C., RIDDLE, N. C. & REED, L. K. 2016. The TreadWheel: a novel apparatus to measure genetic variation in response to gently induced exercise for Drosophila. *PloS one,* 11**,** e0164706.

MILLION, M., MARANINCHI, M., HENRY, M., ARMOUGOM, F., RICHET, H., CARRIERI, P., VALERO, R., RACCAH, D., VIALETTES, B. & RAOULT, D. J. I. J. O. O. 2012. Obesity-associated gut microbiota is enriched in Lactobacillus reuteri and depleted in Bifidobacterium animalis and Methanobrevibacter smithii. 36**,** 817-825.

MOGHADAM, N. N., THORSHAUGE, P. M., KRISTENSEN, T. N., DE JONGE, N., BAHRNDORFF, S., KJELDAL, H. & NIELSEN, J. L. J. F. 2018. Strong responses of Drosophila melanogaster microbiota to developmental temperature. 12**,** 1-12.

MORAIS, P. B., MARTINS, M. B., KLACZKO, L. B., MENDONÇA-HAGLER, L. C. & HAGLER, A. N. 1995. Yeast succession in the Amazon fruit Parahancornia amapa as resource partitioning among Drosophila spp. *Applied and environmental microbiology,* 61**,** 4251-4257.

MURUGESAN, S., ULLOA-MARTÍNEZ, M., MARTÍNEZ-ROJANO, H., GALVÁN-RODRÍGUEZ, F., MIRANDA-BRITO, C., ROMANO, M., PIÑA-ESCOBEDO, A., PIZANO-ZÁRATE, M.,

HOYO-VADILLO, C., GARCÍA-MENA, J. J. E. J. O. C. M. & DISEASES, I. 2015. Study of the diversity and short-chain fatty acids production by the bacterial community in overweight and obese Mexican children. 34**,** 1337-1346.

NEWELL, P. D. & DOUGLAS, A. E. 2014a. Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in Drosophila melanogaster. *Applied and environmental microbiology,* 80**,** 788-796.

NEWELL, P. D. & DOUGLAS, A. E. J. A. E. M. 2014b. Interspecies interactions determine the impact of the gut microbiota on nutrient allocation in Drosophila melanogaster. 80**,** 788-796.

PAIS, I. S., VALENTE, R. S., SPORNIAK, M. & TEIXEIRA, L. J. P. B. 2018. Drosophila melanogaster establishes a species-specific mutualistic interaction with stable gut-colonizing bacteria. 16**,** e2005710.

PARKS, B. W., NAM, E., ORG, E., KOSTEM, E., NORHEIM, F., HUI, S. T., PAN, C., CIVELEK, M., RAU, C. D. & BENNETT, B. J. J. C. M. 2013. Genetic control of obesity and gut microbiota composition in response to high-fat, high-sucrose diet in mice. 17**,** 141-152.

PAWITAN, Y., MICHIELS, S., KOSCIELNY, S., GUSNANTO, A. & PLONER, A. J. B. 2005. False discovery rate, sensitivity and sample size for microarray studies. 21**,** 3017-3024.

PRICE, M. N., DEHAL, P. S., ARKIN, A. P. J. M. B. & EVOLUTION 2009. FastTree: computing large minimum evolution trees with profiles instead of a distance matrix. 26**,** 1641-1650.

PUHL, R. M. & HEUER, C. A. 2010. Obesity stigma: important considerations for public health. *American journal of public health,* 100**,** 1019-1028.

QUAST, C., PRUESSE, E., YILMAZ, P., GERKEN, J., SCHWEER, T., YARZA, P., PEPLIES, J. & GLÖCKNER, F. O. J. N. A. R. 2012. The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. 41**,** D590-D596.

READ, M. N. & HOLMES, A. J. 2017. towards an integrative Understanding of Diet–Host–Gut Microbiome interactions. *Frontiers in Immunology,* 8.

REED, L. K., LEE, K., ZHANG, Z., RASHID, L., POE, A., HSIEH, B., DEIGHTON, N., GLASSBROOK, N., BODMER, R. & GIBSON, G. 2014. Systems genomics of metabolic phenotypes in wild-type Drosophila melanogaster. *Genetics,* 197**,** 781-793.

REED, L. K., WILLIAMS, S., SPRINGSTON, M., BROWN, J., FREEMAN, K., DESROCHES, C. E., SOKOLOWSKI, M. B. & GIBSON, G. 2010. Genotype-by-diet interactions drive metabolic phenotype variation in Drosophila melanogaster. *Genetics,* 185**,** 1009-1019.

RIDAURA, V. K., FAITH, J. J., REY, F. E., CHENG, J., DUNCAN, A. E., KAU, A. L., GRIFFIN, N. W., LOMBARD, V., HENRISSAT, B. & BAIN, J. R. 2013. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science,* 341**,** 1241214.

RIDLEY, E. V., WONG, A. C., WESTMILLER, S. & DOUGLAS, A. E. 2012. Impact of the resident microbiota on the nutritional phenotype of Drosophila melanogaster. *PloS one,* 7**,** e36765.

ROSENBERG, E., SHARON, G., ATAD, I. & ZILBER-ROSENBERG, I. 2010. The evolution of animals and plants via symbiosis with microorganisms. *Environmental microbiology reports,* 2**,** 500-506.

ROSENTHAL, N. & BROWN, S. J. N. C. B. 2007. The mouse ascending: perspectives for human-disease models. 9**,** 993-999.

RULIFSON, E. J., KIM, S. K. & NUSSE, R. J. S. 2002. Ablation of insulin-producing neurons in flies: growth and diabetic phenotypes. 296**,** 1118-1120.

RUSSELL, T., KURTZ, R. J. S. & REVIEWERS-JULY 2012. A Comparison of Laboratory-Reared Stock and Captured Fruit Flies (Drosophila melanogaster) using Upward Movement, Phototaxic, and Starvation Assays Reveals Significant Behavioral Differences. 6.

RYU, J.-H., KIM, S.-H., LEE, H.-Y., BAI, J. Y., NAM, Y.-D., BAE, J.-W., LEE, D. G., SHIN, S. C., HA, E.-M. & LEE, W.-J. 2008. Innate immune homeostasis by the homeobox gene caudal and commensal-gut mutualism in Drosophila. *science,* 319**,** 777-782.

SANG, J. H. J. J. O. E. B. 1956. The quantitative nutritional requirements of Drosophila melanogaster. 33**,** 45-72.

SCHWIERTZ, A., TARAS, D., SCHÄFER, K., BEIJER, S., BOS, N. A., DONUS, C. & HARDT, P. D. J. O. 2010. Microbiota and SCFA in lean and overweight healthy subjects. 18**,** 190-195.

SEGANFREDO, F., BLUME, C., MOEHLECKE, M., GIONGO, A., CASAGRANDE, D., SPOLIDORO, J., PADOIN, A., SCHAAN, B. & MOTTIN, C. 2017. Weight-loss interventions and gut microbiota changes in overweight and obese patients: a systematic review. *Obesity Reviews*.

SGRO, C. M. & PARTRIDGE, L. J. T. A. N. 2000. Evolutionary responses of the life history of wild-caught Drosophila melanogaster to two standard methods of laboratory culture. 156**,** 341-353.

SHIN, S. C., KIM, S.-H., YOU, H., KIM, B., KIM, A. C., LEE, K.-A., YOON, J.-H., RYU, J.-H. & LEE, W.-J. 2011. Drosophila microbiome modulates host developmental and metabolic homeostasis via insulin signaling. *Science,* 334**,** 670-674.

SKORUPA, D. A., DERVISEFENDIC, A., ZWIENER, J. & PLETCHER, S. D. 2008. Dietary composition specifies consumption, obesity, and lifespan in Drosophila melanogaster. *Aging cell,* 7**,** 478-490.

STAUBACH, F., BAINES, J. F., KÜNZEL, S., BIK, E. M. & PETROV, D. A. J. P. O. 2013. Host species and environmental effects on bacterial communities associated with Drosophila in the laboratory and in the natural environment. 8.

STORELLI, G., DEFAYE, A., ERKOSAR, B., HOLS, P., ROYET, J. & LEULIER, F. J. C. M. 2011. Lactobacillus plantarum promotes Drosophila systemic growth by modulating hormonal signals through TOR-dependent nutrient sensing. 14**,** 403-414.

TEFIT, M., GILLET, B., JONCOUR, P., HUGHES, S. & LEULIER, F. 2017. Stable association of a Drosophila-derived microbiota with its animal partner and the nutritional environment upon transfer between populations and generations. *bioRxiv***,** 111492.

TILG, H. & MOSCHEN, A. R. 2016. Gut Microbiome, Obesity, and Metabolic Syndrome. *Metabolic Syndrome: A Comprehensive Textbook***,** 447-459.

TONG, Q. & ZHANG, J.-T. J. F. I. M. 2019. Effects of captivity and season on the gut microbiota of the brown frog (Rana dybowskii). 10**,** 1912.

TURNBAUGH, P. J., LEY, R. E., MAHOWALD, M. A., MAGRINI, V., MARDIS, E. R. & GORDON, J. I. 2006. An obesity-associated gut microbiome with increased capacity for energy harvest. *nature,* 444**,** 1027-131.

VACCHINI, V., GONELLA, E., CROTTI, E., PROSDOCIMI, E. M., MAZZETTO, F., CHOUAIA, B., CALLEGARI, M., MAPELLI, F., MANDRIOLI, M. & ALMA, A. 2017. Bacterial diversity shift determined by different diets in the gut of the spotted wing fly Drosophila suzukii is primarily reflected on acetic acid bacteria. *Environmental microbiology reports,* 9**,** 91-103.

WAHBA, I. M. & MAK, R. H. 2007. Obesity and obesity-initiated metabolic syndrome: mechanistic links to chronic kidney disease. *Clinical Journal of the American Society of Nephrology,* 2**,** 550-562.

WILLIAMS, S., DEW-BUDD, K., DAVIS, K., ANDERSON, J., BISHOP, R., FREEMAN, K., DAVIS, D., BRAY, K., PERKINS, L. & HUBICKEY, J. 2015. Metabolomic and gene expression profiles exhibit modular genetic and dietary structure linking metabolic syndrome phenotypes in Drosophila. *G3: Genes, Genomes, Genetics,* 5**,** 2817-2829.

WONG, A. C.-N., DOBSON, A. J. & DOUGLAS, A. E. 2014. Gut microbiota dictates the metabolic response of Drosophila to diet. *Journal of Experimental Biology,* 217**,** 1894-1901.

WONG, A. C.-N., LUO, Y., JING, X., FRANZENBURG, S., BOST, A. & DOUGLAS, A. E. J. A. E. M. 2015. The host as the driver of the microbiota in the gut and external environment of Drosophila melanogaster. 81**,** 6232-6240.

WONG, A. C., CHASTON, J. M. & DOUGLAS, A. E. 2013. The inconstant gut microbiota of Drosophila species revealed by 16S rRNA gene analysis. *The ISME journal,* 7**,** 1922.

WONG, C. N. A., NG, P. & DOUGLAS, A. E. 2011. Low-diversity bacterial community in the gut of the fruitfly Drosophila melanogaster. *Environmental microbiology,* 13**,** 1889-1900.

WU, G. D., CHEN, J., HOFFMANN, C., BITTINGER, K., CHEN, Y.-Y., KEILBAUGH, S. A., BEWTRA, M., KNIGHTS, D., WALTERS, W. A. & KNIGHT, R. J. S. 2011. Linking long-term dietary patterns with gut microbial enterotypes. 334**,** 105-108.

ZHANG, C., ZHANG, M., WANG, S., HAN, R., CAO, Y., HUA, W., MAO, Y., ZHANG, X., PANG, X. & WEI, C. J. T. I. J. 2010. Interactions between gut microbiota, host genetics and diet relevant to development of metabolic syndromes in mice. 4**,** 232-241.